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A Kinetic Flow Technic for Study of Immune Hemolysis.*† (24575)

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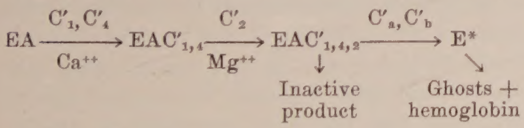
In previous publications from this laboratory(1,2), the lysis of sensitized sheep erythrocytes (EA; where E = erythrocyte, A = antibody to the Forssman hapten) by guinea pig complement (C′) has been shown to proceed according to the following mechanism:



$$\begin{tikzcd} EA \arrow[r, "C'_1, C'_4", "Ca^{++}"'] & EAC'_{1,4} \arrow[r, "C'_2", "Mg^{++}"'] & EAC'_{1,4,2} \arrow[r, "C'_a, C'_b"] \arrow[d] & E^* \arrow[d] \\ & & \text{Inactive product} & \text{Ghosts + hemoglobin} \end{tikzcd}$$

In this scheme, C'_1, C'_2 and C'_4 represent first, second and fourth components of C′ as defined by the conventional component reagents (3), while C'_a and C'_b are 2 components of C′ whose existence has recently been shown by Rapp(2); both are distinct from C'_1, C'_2 and C'_4, and either one or both of them are identical with the third component of C′, *i.e.*, C'_3, as defined in the conventional manner(3). C'_a and C'_b both act in absence of divalent cations. The symbols EA, $EAC'_{1,4}$ and $EAC'_{1,4,2}$ do not denote chemical compounds but intermediate products, *i.e.*, sensitized cells, sensitized cells which have reacted with C'_1 and C'_4, and sensitized cells which have reacted with C'_1, C'_4 and C'_2, respectively. E* represents a distinct intermediate which is capable of lysis without further interaction with C′ components. The present publication describes a technic by which formation and disappearance of each of these intermediates may be visualized in a single experiment. The method represents an extension and refinement of technics employed in our earlier studies of appearance and disappearance of single intermediates(1).

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Materials and methods. Preparation of veronal buffer, sheep erythrocyte (E) suspensions, EA, and of a reagent (C'_y) furnishing C'_2, C'_a and C'_b, as well as general experimental procedures for performing kinetic analyses of immune hemolysis, have been described(1). These were followed with minor modifications to be described in greater detail elsewhere. *Procedures.* A flask containing 6×10^9 EA and 1 ml of a 1/14 dilution of C′ in final volume of 90 ml with Ca^{++} (1.5×10^{-4} *M*) and Mg^{++} (5×10^{-4} *M*) present, was rocked continuously in water bath at 37.0°C. Aliquot samples of 1.5 ml were removed at desired intervals and mixed with 3 ml of ice-cold veronal buffer. They were centrifuged in the cold for 2-5 min. at 1000 g within 1 minute after withdrawal. Supernatant fluids were removed immediately after centrifugation and set aside for hemoglobin analysis, the tubes were drained and the lips wiped with absorbent paper. The sedimented cells were resuspended smoothly in *one* of the following reagents: 1 ml C'_y diluted 1/24 with respect to original serum in veronal buffer containing 10^{-3} *M* Mg^{++} (C'_y-Mg^{++}), 1.0 ml C'_y diluted 1/24 with respect to original serum in veronal buffer containing 5×10^{-3} *M* ethylene diamine tetraacetate (C'_y-EDTA), or 4.5 ml of plain veronal buffer. To retard further reactions of C′ in the samples after withdrawal and during these manipulations, the entire experiment was performed in cold room at 3-7°C. The resuspended cells were further incubated for 1 hour at 37°C. Then 3.5 ml of plain veronal buffer were added to those tubes which had previously received C'_y. All tubes were then centrifuged and the supernatant fluids were diluted ⅓ for hemoglobin determination at 410.5 mμ in a Beckman Model DU spectrophotometer. The supernatant fluids obtained after first centrifugation of samples were treated similarly.

Results. The data from a typical experiment (#100256) are shown in Fig. 1. The ordinates give the proportion of *original* cell population which either had lysed (curve labeled "Original Supernatants") or could be lysed by the reagent indicated (other curves), at time of sampling indicated on abscissae.

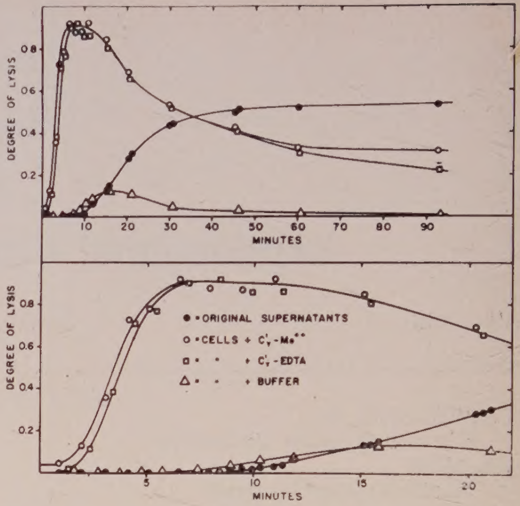

FIG. 1. Formation and disappearance of intermediates in immune hemolysis (Exp. #100256). Top portion: entire reaction. Bottom portion: early part of reaction on magnified time scale. Full explanation appears in text. Symbols have same meaning in both parts of figure. Reagent blanks for C'_y were obtained on samples drawn from flasks containing EA without C′, and subjected to the same treatment as samples from the flask containing EA and C′; avg degrees of lysis (4 determinations) were: .039 with C'_y-Mg^{++}, and .006 with C'_y-EDTA (both corrected for spontaneous lysis in buffer).

Discussion. The differences among the curves obtained by exposing aliquots of cells from the same reaction mixture to different reagents reflect availability or unavailability of the various C′ components in these reagents; thus, plain veronal buffer allows only cells in the state E* to lyse; C'_y-EDTA furnishes C'_a and C'_b, as well as allowing lysis of E*; C'_y-Mg^{++} provides C'_2 in addition to C'_a and C'_b, and also permits lysis of E*. However, it would be erroneous to conclude that these cells lysed by C'_y-Mg^{++}, but not by C'_y-EDTA, are cells "in the state $EAC'_{1,4}$," in the terminology of our previous publications(1). Similarly, the proportion of cells lysed by C'_y-EDTA, after correction for spontaneous lysis of E*, cannot be equated to the proportion of cells "in the state $EAC'_{1,4,2}$." This criticism would also apply to Leon's study of intermediates formed by human C′ (4).

To interpret data of this type correctly, it is necessary to use the "site concept" developed by Rapp(5) and described by Mayer (6). The concept distinguishes between molecular reactions taking place at individual sites on erythrocyte surface and the cellular event of hemolysis which is the consequence of these reactions. Thus, a sensitized site, SA, reacts first with C'_1 and C'_4 to form $SAC'_{1,4}$, and then with C'_2 to yield $SAC'_{1,4,2}$; the latter site either decays or reacts with C'_a and C'_b to become S*. A cell with at least 1 S* is in the state E*. Mathematical analysis of this model indicates that the proportion of cells lysed depends non-linearly on average number of $SAC'_{1,4,2}$ per cell and on concentration of C'_a and C'_b to which the sample of cells is exposed. Thus, the proportion of cells found to be "in the state $EAC'_{1,4,2}$" in the present system would vary with concentration of C'_a and C'_b in the reagent, making it impossible to interpret the results in these terms.

In terms of "site concept" then, the meaning of curves in Fig. 1 is as follows: curve labeled "Original Supernatants" shows progress of lysis; curve labeled "Cells + Buffer" shows proportion of E*; curve labeled "Cells + C'_y-EDTA" shows proportion of E* plus proportion of cells lysed as a result of the presence of $SAC'_{1,4,2}$, the difference between this curve and the "Cells + Buffer" curve being a measure of the average number of $SAC'_{1,4,2}$ per cell; curve labeled "Cells + C'_y-Mg^{++}" shows proportion of E* plus proportion of cells lysed as a result of the presence of $SAC'_{1,4,2}$ plus proportion of cells lysed as a result of the presence of $SAC'_{1,4}$ on the same cells, which are converted to $SAC'_{1,4,2}$ by the C'_2 furnished by the reagent. Thus, the difference between the "Cells + C'_y-Mg^{++}" and "Cells + C'_y-EDTA" curves is a measure of the average number of $SAC'_{1,4}$ per cell, except where the 2 curves coincide as a result of the presence of a sufficient number of $SAC'_{1,4,2}$ per cell to yield virtually complete hemolysis with concentration of C'_a and C'_b added, in which event formation of further $SAC'_{1,4,2}$ from $SAC'_{1,4}$ by reaction with the C'_2 added with the reagent makes no difference.

The technic here described furnishes a means whereby C′ from different species may be compared(7) and the effect of C′ inhibitors studied(8) without recourse to use of conventional reagents for the C′ components(3), to which serious objections have been stated by Mayer(6). These and other applications are presently under study in this laboratory.

Summary. A method for the kinetic analysis of the formation and disappearance of intermediates in immune hemolysis has been described. The interpretation of results obtained with it has been discussed, and certain applications indicated.

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Placental Transfer and Biological Half-life of Radioactive Vit. B_{12} in the Dog.* (24576)

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Little is known about placental transfer of Vit. B_{12} in humans. In the rat, Chow *et al.* studied placental transfer using radioactive $Co^{60}B_{12}$ as tracer. They found that after subcutaneous injection into pregnant rats, 12% of 200 mμg dose(1) and in later experiments, 60% of 10 μμg dose(2) was transferred to the litter. Rice *et al.*(3) also reported on placental transfer of $Co^{60}B_{12}$ in the rat, showing average of 2.3% of 3 μg orally administered dose given to pregnant rats, was transferred to the fetuses. We investigated the placental transfer and biological half-life of $Co^{60}B_{12}$ in the dog, during studies of the physiology of Vit. B_{12} in human maternal and fetal nutrition. The dog was chosen in preliminary investigation of certain technical problems anticipated in the study of human maternal-fetal Vit. B_{12} metabolism. Some of these results are reported herein.

Methods and materials. A 16 lb. bitch of mixed breed was given subcutaneous dose of 9.6 μc Vit. $Co^{60}B_{12}$ equivalent to 9.6 μg Vit. B_{12}, 5 months prior to mating and approximately 7 months prior to delivery of her litter. She nursed the litter of 4 pups until weaned at 4 weeks of age. Uptake and retention of radioactivity was studied by external scanning over the hepatic area. A scintillation probe-type counter employing 1″ x 1″ NaI thallium-activated crystal was used for external radioactivity scanning. Hepatic surface radioactivity in counts/min. (CPM) was determined 3 times a week on each dog. "Shielded" background radioactivity was determined by scanning the left lower leg. Absolute radioactivity (body cpm—shielded background cpm) were corrected for physical decay of Co^{60}. The mother and pup No. 1 were followed by surface scanning of radioactivity for 6 weeks post partum. At this time they were sacrificed and certain organs obtained. The tissues were cleaned, homogenized in Waring blender with measured amounts of water, and 3 weighed aliquots of each homogenate counted in duplicate for radioactivity in well-type NaI scintillation counter. Radioactivity counts were equated to microcuries of Vit. $Co^{60}B_{12}$ by comparison with known standard $Co^{60}B_{12}$ solution counted under identical conditions. Pups No. 2 and 3 were given small parenteral "calibration" doses of $Co^{60}B_{12}$, 4 weeks after birth. By determining increase in external hepatic area counts due to "calibration" dose, it was possible to estimate(4) the amount of Vit. $Co^{60}B_{12}$ transferred placentally. Pup No. 4 was continued on routine diet until all externally detectable radioactivity disappeared.

Results. Placental transfer of $Co^{60}B_{12}$ estimated from hepatic radioactivity. Data for calculation of amount of Vit. B_{12} transferred placentally as determined by external hepatic area radioactivity scanning technic, are presented in Table I. The 2 small physiologic doses of 0.0385 and 0.116 μc $Co^{60}B_{12}$ (μc = μg) were assumed to be completely retained. From the increment in cpm over the hepatic area after the calibration dose, it was calculated that millicuries/cpm were 0.208 and 0.172 for pup Nos. 2 and 3, respectively. Therefore, counts of 390 and 400 at birth represent 0.081 and 0.069 μg $Co^{60}B_{12}$ transferred placentally. Thus 3-4% of initial subcutaneous dose given to the bitch was transferred to the 4 pups.

Tissue distribution of placentally transferred $Co^{60}B_{12}$. Distribution of $Co^{60}B_{12}$ in certain major organs of the bitch and pup No. 1 are presented in Fig. 1. In tissues studied, concentration (μμg/g) in the bitch was highest in the kidney followed in order by spleen, pancreas, liver and small intestine. However, by far the greatest total amount of radioactivity resided in the liver because of its much greater weight. Similarly, in the pup, the kidney contained the highest concentration of $Co^{60}B_{12}$ although the liver con-

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TABLE I. Placental Transfer of $Co^{60}B_{12}$ in the Dog. Calculated from external hepatic area. Radioactivity (EHAR) scanning.

	EHAR at birth (avg cpm)	"Calibration" dose (μc $Co^{60}B_{12}$)	Increment of EHAR (avg cpm)	$m\mu c/cpm$	μg $Co^{60}B_{12}$ placentally transferred
Pup 2	390	.0385	185	.208	.081
3	400	.116	670	.172	.069

tained the largest amount of this vitamin. The proportional distribution of $Co^{60}B_{12}$ among liver, kidneys and pancreas for both dogs was similar.

Placental transfer from tissue distribution of $Co^{60}B_{12}$. The above characteristic permits calculation of placentally transferred $Co^{60}B_{12}$ by comparison of individual organ content of $Co^{60}B_{12}$ in the mother dog with that in her pups. Such data are presented in Table II, from which it is evident that an average of 5.1% of radioactive $Co^{60}B_{12}$ *retained* by the mother was placentally transferred to the pup. Since externally detectable radioactivity over the hepatic area at birth was similar for the other 3 pups, it is reasonable to assume that approximately 20% of mothers B_{12} goes to the entire litter.

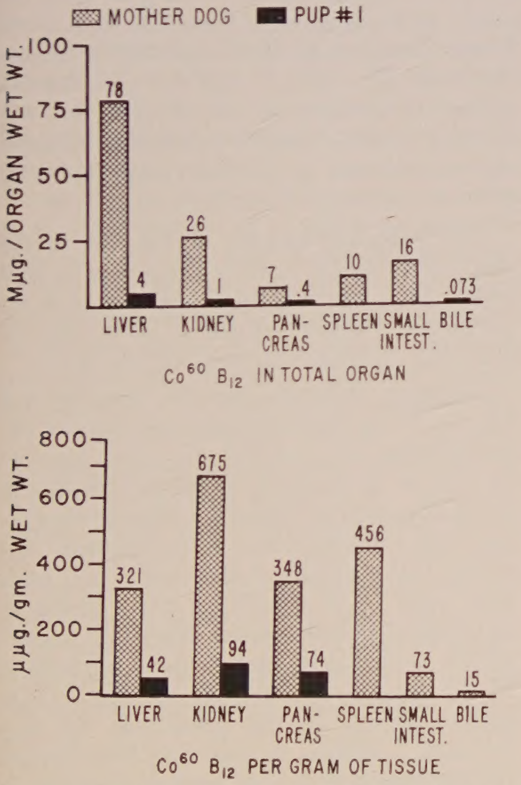

FIG. 1. Tissue distribution of $Co^{60}B_{12}$ in the dog: Comparison of mother dog with that placentally transferred to her pup.

Biological half-life of $Co^{60}B_{12}$. Biological elimination of radioactive $Co^{60}B_{12}$ was studied in all dogs, by following disappearance rate of externally detectable radioactivity over the hepatic area, measured in carefully standardized manner so that the relative geometry was always essentially the same. The percentage of counts over the hepatic area decreased in all pups and the bitch at a similar rate, regardless of number of counts in the particular animal. The time for radioactivity to decrease by half varied from 48 to 70 days (Fig. 2). This indicates a biological half-life for Vit. B_{12} of 1.6 to 2.3 months in the post partum adult dog and pups.

Discussion. Two technics were employed for study of placental transfer of $Co^{60}B_{12}$ in the dog: (a) calibration of externally detectable hepatic radioactivity in the newborn animal, and (b) comparison of organ content of $Co^{60}B_{12}$ in mother and pup. The dog lends itself well to the first method because of its relatively large size. In this respect, the technical situation is akin to that in human newborns.

Determination of extent of placental transfer by comparison of $Co^{60}B_{12}$ content of similar organs in mother and newborn is a novel method, capable of great accuracy, and is

TABLE II. Placental Transfer of $Co^{60}B_{12}$ in Dog. Calculated from comparison of individual organ tissue content of $Co^{60}B_{12}$ in mother dog and her pup.

	$m\mu g$ $Co^{60}B_{12}$			% of total $Co^{60}B_{12}$ transferred to pup
Organ	Mother	Pup	Total	
Liver	77.8	4.3	82.1	5.23
Kidney	26	1.25	27.3	4.6
Pancreas	7.3	.4	7.7	5.2
				5.1 Avg

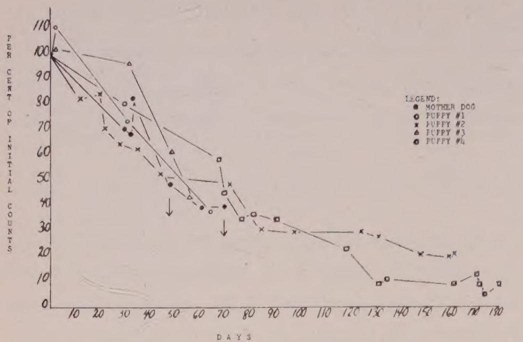

FIG. 2. Biological half-life of $Co^{60}B_{12}$ in post partum dog and puppies.

especially useful in large animal species where there is difficulty in determining B_{12} content of the entire carcass. For this comparison to be a true reflection of physiologic placental transfer, it is essential that Vit. B_{12} be administered and distributed in the mother's organs before gestation begins. From our data in humans(5,6) it appears that administration of B_{12} during pregnancy floods the blood stream and a considerable portion of these newly introduced B_{12} molecules are taken up by the placenta and fetus.

It is significant that concentration of Co^{60} B_{12} in liver, kidney and pancreas of the newborn pup was ⅕ to ⅛ that of corresponding maternal tissue (Fig. 1).

From data of Ross and Mollin(7) on total Vit. B_{12} content of liver in human infants, it is apparent that concentration of B_{12} in the liver of human newborns is in the same proportional range to adult liver as we found in the dog. The finding that maternal plasma Vit. B_{12} level falls progressively throughout pregnancy, reaching subnormal levels at term, and plasma Vit. B_{12} level of the newborn is very high, approaching 2 to 5 times that of the corresponding maternal value(8) has been explained by many workers(2,3,9) as indicating that the fetus parasitizes and depletes the maternal stores of Vit. B_{12}. The recently reported increased gastrointestinal absorption of Vit. B_{12} during normal pregnancy(2,3) also supports the latter inference. However, huge unphysiologic doses of Vit. B_{12} were used in some of the latter experiments, and it is questionable whether such data reflect the status of absorption of dietary amounts of B_{12}.

An alternate explanation for the physiology of maternal-fetal Vit. B_{12} interrelationships may be suggested from our data. The progressively decreasing Vit. B_{12} plasma level during pregnancy may be explained by our previously published finding(5,6) that (a) gastrointestinal absorption of dietary amounts of Vit. B_{12} is diminished in the last trimester of pregnancy, and that (b) a large part of Vit. B_{12} introduced into the maternal blood stream during latter part of pregnancy (whether after gastrointestinal absorption or following parenteral administration) is selectively distributed to the placenta and fetus in addition to the maternal tissues. The fetal parasitization of the mother may thus be limited to Vit. B_{12} in the mother's "mobilizable stores" which in turn is possibly highly dependent upon her dietary intake rather than her tissue stores which may be tightly bound.

The high Vit. B_{12} levels in the newborn, on the other hand, do not appear to reflect increased accumulation of Vit. B_{12} in fetal tissues. An explanation of these high fetal levels is not forthcoming from currently available data, but it is probable that this phenomenon is due to a characteristic of placental B_{12} transfer system. Such a mechanism explains the maintenance of a similar maternal fetal plasma gradient for riboflavin(10).

The biological half-life of Vit. B_{12} in the dog is surprisingly short in comparison to that in humans. The dog may thus be a good experimental subject for study of factors involved in biological turnover of Vit. B_{12}.

Summary. Placental transfer of radioactive Vit. B_{12} in the dog following pregestational administration, was found in the entire litter to be 20% of the residual radioactive vitamin in the bitch at time of delivery. Because the proportional distribution of radioactive B_{12} among major organs of the mother and pups was found to be similar, it was possible to calculate placental transfer from this characteristic as well as by postpartum "calibration" of the hepatic radioactivity in pups. Biological half-life of the radioactive Vit. B_{12} averaged 2 months in pups and bitch during postpartum period. A new explanation is suggested for the low maternal and high fetal plasma levels of Vit. B_{12}.

We thank Drs. G. B. Jerzy Glass and W. Mersheimer for initiating these studies and D. N. Teller, J. M. Guidi, and J. B. Wenig for invaluable technical assistance.

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Total, Phospholipid and Labile Phosphorus in Serum and Tissue Employing Chloric Acid and N-Phenyl-P-Phenylenediamine.* (24577)

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The advantages of chloric acid as a reagent suitable for digestion of serum and serum extracts in the colorimetric estimation of phosphorus have been pointed out(1). Its rapidity, ease of digestion and of handling has resulted in considerable reduction of digestion time. Our purpose is to report an improvement in the chloric acid procedure which provides a less tedious method for color development thus rendering the method more desirable for routine determinations. In most procedures the inorganic phosphate present after digestion is treated with molybdate forming a phosphomolybdate complex. Addition of a suitable reducing agent selectively reduces this complex to yield a blue color. Many reducing agents have been investigated in an effort to find one which will give a stable color in the presence of the neutralized decomposition products of chloric acid digestion. Stannous chloride(2,3,4), 1,2,4-aminonaptholsulfonic acid(5) 2,4-diaminophenol(6), p-methyl aminophenol sulfate(7,8) and ferrous sulfate (9) all proved to be unsatisfactory with chloric acid digests. Hydrazine sulfate(1) has proved a sensitive reductant, but requires heating for color development. Use of p-semidine hydrochloride(10) eliminates this step.

Procedure for phospholipid phosphorus in serum. Chloric acid reagent. 500 g of potassium chlorate in a 3 liter beaker with 900 ml of distilled water was heated until solution was effected. After cooling, 375 ml of 72% perchloric acid was added with constant stirring. Some potassium perchlorate precipitated; the covered beaker was kept in a refrigerator for 12-24 hours, then filtered with suction through Whatman 41H filter paper. The filtrate was an approximately 28% solution of chloric acid. *Standard phosphorus solution stock.* Pure dry mono potassium phosphate, 0.351 g was dissolved in distilled water. Ten ml of 10N sulfuric acid was added and diluted to 1 liter with water. This solution contained 0.08 ml of phosphorus per ml. *Working standard phosphorus solution.* 50 ml of the standard phosphorus stock solution was diluted to 200 ml with water. Each ml contained 0.02 mg of phosphorus. *Molybdate reagent.* 0.024 M ammonium molybdate in

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10N sulfuric acid. *p-Semidine hydrochloride.*[†] 50 mg in 100 ml of 1% sodium bisulfite. After vigorous shaking the small amount of insoluble residue was filtered. *Phenolphthalein indicator.* One per cent solution of phenolphthalein in 95% ethanol. *8% sodium hydroxide in water. Alcohol-ether mixture.* Three volumes of 95% reagent ethanol and one volume of reagent grade diethyl ether were mixed. *Extraction.* 1 ml of serum was slowly pipetted into a 25 ml volumetric flask containing approximately 18 ml of alcohol-ether mixture. This was stoppered loosely, placed in a boiling water bath and brought to a boil. Cooled and diluted to the 25 ml mark. The mixture was filtered rapidly to avoid loss of solvent by evaporation. *Digestion.* 5 ml aliquots of the filtrate were evaporated to dryness in a 180 ml electrolytic beaker. Two or 3 acid washed Henger crystals were put into the beaker, along with 5 ml of chloric acid, and 4 drops of 10% NaCl. The beaker was covered with a borosilicate watch glass and digested on a hot plate till white fumes of perchloric acid appeared. At this point the watch glass was removed and the residue heated to incipient dryness. The beakers were removed from the hot plate and the crystalline residue redissolved in 5 ml of water, washing down the walls of the container. The beaker was placed back on the hot plate and boiled for 2-3 minutes without cover and cooled to room temperature. *Color development.* The phosphate containing solution was neutralized with 8% sodium hydroxide, and a drop of 1% phenolphthalein as indicator. After transfer to a 25 ml volumetric cylinder, 2 ml of molybdate reagent, and 6 ml of p-semidine reagent were added and the volume brought to 20 ml with water. The absorbance was measured after 20 minutes at 700 mμ in a Coleman spectrophotometer. *Standards.* One-half, one and 2 ml portions of the working phosphorus standard solution were pipetted into 180 ml electrocytic beakers. Four drops of 10% sodium chloride were added and digestion and color development proceeded as above. Water carried through the determination was used a blank. *Procedure for total serum phosphorus and organic phosphorus.* To 0.2 ml of whole serum or suitable sample containing 10 to 80 γ of phosphorus in a 180 ml electrocytic beaker, 7 ml of chloric acid was added; digestion and color development proceeded as above. *Procedure for labile phosphorus.* The sample to be analyzed (containing 8 to 80 γ of hydrolyzable phosphorus in one ml or less) was deproteinized by adding 5 ml of 5% trichloroacetic acid. After centrifuging at 2500 rpm for 15 minutes, 1 ml of 5N sulfuric acid was added to the supernatant solution. The tubes were heated in a boiling water bath for 5 to 20 minutes, and cooled. To a 5 ml aliquot, in a 25 ml volumetric cylinder, 1.35 ml of 10N H_2SO_4 was added and mixed. Two ml of 0.024M ammonium molybdate and 6 ml of p-semidine reagent was added and the volume brought to 20 ml with water. The absorbance was measured after 20 minutes at 700 mμ in a Coleman Jr. Spectrophotometer.

Results. Serum samples of donors selected at random at the time of blood donations were estimated for phosphorus content by this

TABLE I. Comparison of Methods for Phospholipid Phosphorus in Blood Serum Using Chloric Acid, 18 Samples.

(mg of phosphorus/100 ml)

Hydrazine reduction method at 830 mμ (Beckman D.U. spectrophotometer)	p-Semidine reduction method at 700 mμ (Coleman Jr. spectrophotometer)	% diff.
7.2	7.2	.00
10.8	10.9	.93
8.0	8.0	.00
8.2	8.2	.00
10.6	10.6	.00
8.0	8.0	.00
6.8	6.6	–2.94
6.4	6.4	.00
7.9	8.0	1.26
8.9	8.8	–1.11
6.5	6.5	.00
8.3	8.2	–1.32
8.5	8.4	–1.17
8.3	8.5	2.40
10.5	10.3	–1.90
8.3	8.3	.00
9.6	9.5	–1.04
9.4	9.1	–3.20
8.46 ± 1.326*	8.42 ± 1.315*	

* Mean and stand. dev.

[†] Obtainable from Distillation Products Industries, division of Eastman Kodak Co., Rochester, N. Y. as N-phenyl-p-phenylenediamine hydrochloride, #2043.

TABLE II. Comparison of Methods for Total Phosphorus in Blood Serum Using Chloric Acid, 12 Samples.

(mg of phosphorus/100 ml)		
Hydrazine reduction method at 830 mμ (Beckman D.U. spectrophotometer)	p-Semidine reduction method at 700 mμ (Coleman Jr. spectrophotometer)	% diff.
13.80	14.00	1.45
14.50	14.00	–3.45
13.00	12.80	1.54
10.30	10.35	.48
13.50	13.00	–3.70
12.50	12.50	.00
14.00	13.80	–1.43
13.80	14.40	4.35
12.60	13.10	3.97
12.50	12.50	.00
13.00	13.00	.00
13.60	13.80	1.47
13.09 ± 1.086*	13.10 ± 1.072*	

* Mean and stand. dev.

method. Chloric acid digestion(1) compares well with the Fiske-Subbarow(5) method for phosphorus estimation. Duplicate samples were determined in establishing values using both methods. The results are shown in Tables I and II.

Discussion. The color obtained by p-semidine reduction is stable, although the rapidity of maximal color development in chloric acid digests is retarded when freshly prepared reagent is not used. Freshly prepared solutions of p-semidine gave maximal color development in 8-12 minutes, the 2-day-old reagent in 12 to 14 minutes, and 10-day-old reagent 20-25 minutes. Therefore, whenever possible this reagent should be prepared immediately prior to use. Filtration of the p-semidine reagent after mixture with one per cent sodium bisulfite is not essential, provided the powdered drug is dispersed sufficiently by thorough shaking in a machine such as a Burrell wrist-action shaker. The insoluble particles remaining do not interfere with color development and in most cases the unfiltered reagent remained stable for a longer period. (Instability was indicated by a change in color to yellow.)

During the digestion process it is necessary that all of chloric acid be destroyed, since it alters color development (the blue reduced phosphomolybdate complex changes to greenish blue). This is accomplished by taking the digest almost to dryness followed by boiling the residual salts in a small amount of water. Reproducibility of the standards is enhanced by addition of 1 or 2 drops of 10% sodium chloride. The sodium chloride probably prevents formation of pyrophosphate during the stage when the digest is brought almost to dryness. The naturally occurring salt in blood serum should have a similar protective influence.

The principal advantages of this method are the relative ease of digestion with chloric acid and the ease of color development and stability of color formed by p-semidine reduction of the phosphomolybdate complex. The phosphorus assay described has been used by us to measure hexokinase and phosphoglucomutase activity in tissue extracts.

Summary. A method is described for determination of serum total and organic bound phosphorus using chloric acid digestion and subsequent formation and reduction of a phosphomolybdate complex with p-semidine. The advantages of the method are ease of digestion and color development and stability of color-complex. This method compares well with the Fiske-Subbarow method.

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Influence of Splenectomy on Resistance to Pneumococcal Infection in Rats. (24578)

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Recent clinical reports have suggested that there is enhanced susceptibility to infection in splenectomized infants and children. King and Shumacker(1) reported 5 grave infections among splenectomized infants. Smith *et al.* (2) described 19 cases of severe infection in children following splenectomy; however, many of their patients had Cooley's anemia, a chronic debilitating disease. In Gofstein and Gellis' analysis(3) of 107 splenectomized children, there were only 4 cases of severe infection. Furthermore, the experience of others does not support the concept of enhanced susceptibility to infection in the splenectomized child. Doan(4) noted only 3 severe infections among 87 children who had splenectomy. Likewise Pierce (personal communication) and Walter and Chaffin(5) have seen no evidence of increased susceptibility to infection in their respective series of splenectomized infants and children. It is difficult to draw definite conclusions from uncontrolled clinical experiences. In animals, it is well known that splenectomy may convert certain latent parasitic infections into manifest disease—for example, *Bartonella muris* in rats, and *Eperythrozoan coccoides* in mice(6). The recently splenectomized monkey has greatly enhanced susceptibility to malarial infection(7). But with respect to the common bacterial infections, the issue is less clear-cut. Most of the work on animals was done before the prevalence and importance of latent bartonellosis in laboratory animals was realized(6). The availability of a Bartonella-free strain of rats in our laboratory made possible an experimental approach to the problem.

Materials and methods. The plan of the study was to compare the response of intact and splenectomized rats to varying doses of intraperitoneally injected bacteria. CF Nelson rats were used. The Bartonella-free nature of the colony was first demonstrated by performing splenectomies on 30 weanling rats. A month later, all animals appeared well, and tail blood was examined. Hematocrits and reticulocyte counts remained within normal limits, and no intra-erythrocytic organisms were seen. The bacterium used was type I pneumococcus originally derived from a patient with otitis media. Lyophilized ampuls containing the organism were broken and contents grown out in trypticase soy broth at 37°C; thereafter, virulence and capsule formation were enhanced by serial intraperitoneal inoculation of RF mice and reculturing from heart blood of moribund animals. Heart blood from third passage was cultured in brain-heart infusion at 37° for 10 hours. For inoculation, serial dilutions were made in brain-heart infusion at room temperature. Assay of inoculum was made by colony count on the surface of blood agar plates. *Exp. 1.* Forty-five suckling rats, 6 to 11 days old, were splenectomized under ether anesthesia, while 51 littermate controls were subjected to laparotomy. Approximately 10 days later, when animals were 17 to 20 days old, they were challenged, all on the same day, by intraperitoneal injection of graded dilutions of the 10-hour 37° culture of pneumococci. For each dosage of inoculum, littermate controls were used. Each family including mother was housed in a separate cage; it was demonstrated that under these conditions there was no cross-infection of non-inoculated rats from animals who succumbed to infection. Animals were checked 3 times daily; those who died were autopsied and cultures were made from the heart blood. Leucocyte counts were made from tail blood by conventional methods, except that 0.6 times the usual volume of blood was used. *Exp. 2* was similar to Exp. 1. Forty-three rats were splenectomized and 42 were laparotomized. Operations were performed at age 5 to 8 days, and bacterial challenge was made on a single day approximately 2 weeks after operation when animals were 19 or 20 days old.

Results. In both splenectomized and con-

TABLE I. Mortality of Intact and Splenectomized Suckling Rats after Intraperitoneal Infection with Pneumococci.

	Dilution of inoculum	Calculated No. bact. per animal	Splenectomized			Control		
			Surv.	Dead	Cumulative % dead*	Surv.	Dead	Cumulative % dead*
Exp. 1	$10^{6.5}$	12.6	0	2	100	0	3	100
	10^{7}	4	0	8	100	0	8	100
	$10^{7.5}$	1.3	3	7	84.2	5	7	78.3
	10^{8}	.4	5	6	53	3	9	57.9
	$10^{8.5}$	.13	5	2	18.8	7	2	11.8
	10^{9}	.04	6	1	5	7	0	0
Exp. 2	10^{7}	23	0	10	100	1	8	95.8
	10^{8}	2.3	1	7	94.1	1	9	88.2
	$10^{8.5}$	.73	6	4	56.2	3	4	54.5
	10^{9}	.23	5	4	29.4	7	1	14.3
	$10^{9.5}$	.07	5	1	5.6	7	1	5

* Calculated by method of Reed and Muench.

trol groups, animals were found dead between 44 and 96 hours after inoculation. Autopsies revealed peritonitis in most and hemorrhagic pneumonitis in about half. In all animals who succumbed, pneumococci were cultured from heart blood.

Mortality data are presented in Table I. In Exp. 1, mean survival time of 26 splenectomized animals that died was 60.1 hours; that of the 29 dead controls was 58.6 hours. The LD_{50} (calculated by method of Reed and Muench) for splenectomized animals was 0.36 bacteria; for the controls, it was 0.33 bacteria. In Exp. 2, the mean survival time of the 26 dead splenectomized animals was 65.7 hours; that of the 23 dead controls was 56.3 hours. The LD_{50} for splenectomized animals was 0.64 bacteria; for controls, 0.56 bacteria.

There was no significant difference in survival time of animals given the higher and lower dilutions of bacterial inoculum. The low LD_{50} values are attributed to the combination of high virulence of the infecting organism and inefficiency of the colony counting as a measure of viable bacteria.

It is concluded that under conditions of this experiment, there is no demonstrable influence of splenectomy on resistance to pneumococcal infection in young rats.

Leucocyte counts. In *Exp. 1,* white cell (WBC) counts from tail blood were performed on survivors 2 weeks after bacterial challenge. The mean WBC of the splenectomized group was 16,500 ± 618, that of controls 12,120 ± 395 (for this difference, $p < 0.1\%$). In *Exp. 2,* WBC counts were made on all animals 3 days before and one day after bacterial inoculation; but data are analyzed only for those animals who subsequently died of infection. For the splenectomized group, mean WBC was 17,370 before infection and 13,800 after infection. For controls, mean WBC was 10,910 before infection and 8,370 after infection. Thus there was some evidence of a post-splenectomy leukocytosis, but no evidence of an extreme leukocytosis in response to infection, such as has been observed occasionally in patients after splenectomy.

Discussion. Pneumococcus was selected for these experiments because it is the organism most frequently reported in clinical post-splenectomy infections. Immature suckling animals were used because of the impression that for resistance to infection the spleen is most important in early life, and because of claims of special susceptibility to infection in the splenectomized infant. Nevertheless, these experiments fail to reveal any difference in resistance to acute infection between normal and splenectomized subjects. It is, of course, possible that such a difference might have been demonstrated with a less virulent strain of bacteria, or with another organism, host, or route of infection. However, lack of good experimental evidence, and conflicting clinical reports render the concept of enhanced susceptibility to bacterial infection in splenectomized subjects open to question.

It is of interest that convincing demonstration of a role of the spleen in resistance has thus far been demonstrated only for infections in which intra-erythrocytic parasitism is prominent(6). Conceivably this situation is related to the function of the spleen in removing abnormal red cells and their inclusion bodies(8).

Summary. No difference in susceptibility to infection by a highly virulent strain of pneumococci could be demonstrated between normal and splenectomized suckling rats. The implications of this finding are briefly discussed.

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Antimetabolite Activity of Lysine Analogues on Lysine Incorporation into Rat Bone Marrow Protein *in vitro*. (24579)

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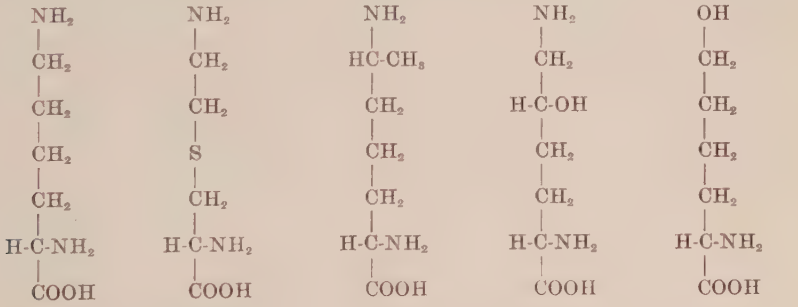
Lysine deficiency in the young rat is attended by anemia(1) brought on by retarded development of the hematopoietic system(2). Recently, Rohdenberg(3) reported that rats fed a lysine-deficient ration accumulated abnormal levels of fat in their bone marrow. It is apparent that integrity and activity of this tissue is markedly sensitive to lysine deprivation. As part of a study on action of amino acid analogues on protein synthesis by isolated mammalian tissues, we investigated the effect of 4 lysine analogues on *in vitro* incorporation of this amino acid into proteins of bone marrow.

Materials and methods. Rats of both sexes maintained on Purina Lab. Chow and weighing 90 to 125 g were decapitated and their femurs rapidly removed and placed in chipped ice. Twenty-five rats furnished sufficient bone marrow for 12 flasks in each experiment. Both ends of bone were cut with scissors and the bone marrow removed by forcing ice cold Krebs Ringer Phosphate (KRP) buffer through the bone with #23 hypodermic needle. The buffer, which was also used as incubation medium, contained .12 M sodium chloride, 1.3 x 10^{-2} M potassium chloride, 1.8 x 10^{-3} M calcium chloride, 6.5 x 10^{-4} M magnesium sulfate, and 0.01 M sodium phosphate, pH 7.2. This procedure dissociated most of the tissue into free cells. The fraction which remained intact was passed through 60 mesh, monel metal sieve and then combined with the free cells. The bone marrow cells were washed 3 times with cold KRP by alternate suspension and sedimentation in refrigerated centrifuge at 2°C and were then resuspended in 2 volumes of buffer. S-(β-Aminoethyl)-L-cysteine hydrochloride had been synthesized by the method of Cavallini *et al.*(4) and was a gift of Dr. F. Tietze of Nat. Inst. for Arthritis and Metabolic Diseases. The ε-C-methyl lysine (2,6-diamino heptanoic acid) was prepared as the dihydrochloride according to the method of McLaren and Knight(5). The δ-hydroxylysine hydrochloride was purchased from Calif. Corp. for Biochemical Research, Los Angeles. The latter 2 analogues were present in their 4 diastereoisomeric forms. Hexahomoserine was prepared as described by Gaudry(6). DL-Lysine-1-C^{14} was a gift of Dr. M. Rothstein, Univ. of California, Berkeley, which had been prepared by a new method(7). Solutions of all compounds were prepared in KRP at pH 7.2. The main compartment of Warburg flasks contained the analogue at a single concentration and DL-Lysine-1-C^{14} at several concentrations. Buffer was added to adjust volume of main compartment to 1.5 ml. One-half ml of bone marrow cells in 2 volumes of buffer was added to one side arm and 0.05 ml of 40% trichloroacetic acid (TCA) was added to second sidearm. After temperature equilibration at 37.5°C for 5 minutes, bone marrow was tipped into the main compartment and incubated 15 minutes. The contents were mixed with TCA to terminate the incorporation. Precipitated material was washed 3 times with 5% TCA, once with 5% TCA at 90°C for 15 minutes, once with 5% TCA at room temperature, once with absolute ethanol, 3 times with 3:1 ethanol-ether mixture at 62°C for 5 minutes, and twice with absolute ether. The protein was plated on aluminum discs and counted in flow-gas counter.

Results. Of the 4 analogues of lysine tested only 2, S-(β-aminoethyl)-cysteine and ε-C-methyl lysine, were effective competitive inhibitors of lysine incorporation into bone marrow protein. These 2 analogues have been shown to be effective lysine antagonists in bacterial growth studies(8,5). Double reciprocal plots showing characteristics of their inhibition are shown in Figs. 1 and 2. The kinetics resemble those found for the action of phenylalanine analogs(9) and ethionine(10) on incorporation of their corresponding metabolites into Ehrlich ascites tumor protein. Inhibition indices (mol analogue/mol lysine for 50% inhibition) estimated on basis of L forms of both analogs and lysine present, were 3 for S-(β-aminoethyl)-cysteine and 20 for ε-C-methyl lysine.

Hexahomoserine was found by Gaudry and co-workers(11) to induce anemia when fed to rats and thus served as basis for explanation of a similar syndrome(12) in rats fed diazotized casein. Prevention of anemia by dietary lysine suggested that hexahomoserine was a potent lysine antagonist(11). It is of interest that this compound was not a lysine antagonist in the *in vitro* system (Fig. 2). We found similar completely negative results when lysine incorporation into hemoglobin of rabbit reticulocytes was used as test system. It is probable that toxic manifestations observed when hexahomoserine is included in rations of rats is brought about by a systemic effect, such as an amino acid imbalance and that the results should not be interpreted to mean that the compound is a lysine antagonist.

Not only does substitution of a hydroxy for an amino group result in a non-inhibitory analog, but the presence of a hydroxy group on the penultimate carbon of lysine also produces an ineffective analog. Thus, δ-hydroxylysine did not competitively inhibit lysine incorporation. This analog produced a non-competitive inhibton of about 30% for all levels of lysine tested (Fig. 3). A similar inhibition by δ-hydroxylysine was earlier observed under identical conditions with leucine incorporation into Ehrlich ascites tumor protein(13). The latter inhibition was an indirect result of the action of δ-hydroxylysine as an inhibitor of



Compound	lysine	S-(β-aminoethyl) cysteine	ε-C-methyl lysine	δ-hydroxylysine	hexahomoserine
Antimetabolic action		very active, competitive	moderately active, competitive	low activity, not competitive	inactive
Inhibition index		3	20		

glutamine synthesis; and it is possible that it may also act in this manner with bone marrow.

The results are summarized in the accompanying tabulation.

Summary. Four lysine analogs were studied for antimetabolic action against radioactive lysine incorporation into rat bone marrow protein *in vitro.* S-(β-Aminoethyl)-cysteine was a potent competitive inhibitor and ϵ-C-methyl lysine was moderately active. δ-Hydroxylysine inhibited slightly and in a non-competitive manner. Hexahomoserine was inactive.

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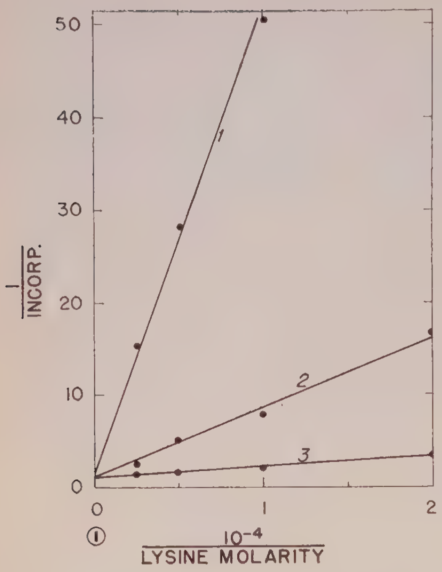

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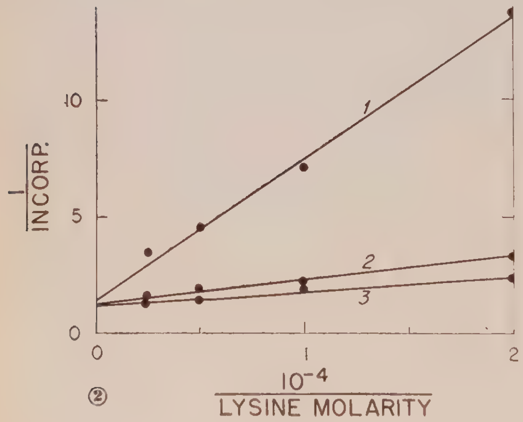

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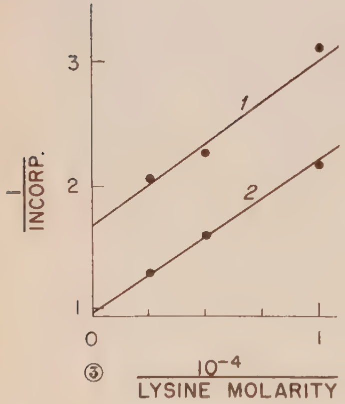

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FIG. 1. Double reciprocal plot showing competitive inhibition by S-(β-aminoethyl)-cysteine of lysine incorporation into bone marrow protein. Curve 1, inhibition by S-(β-aminoethyl)-cysteine, 5×10^{-3} M; Curve 2, inhibition by S-(β-aminoethyl)-cysteine, 1×10^{-3} M; Curve 3, uninhibited incorporation. Incorporation expressed as lysine incorporated/g of protein during 15-min. incubation period.

FIG. 2. Double reciprocal plot showing competitive inhibition by ϵ-C-methyl lysine and hexahomoserine of lysine incorporation into bone marrow protein. Curve 1, inhibition by ϵ-C-methyl lysine, 1×10^{-2} M; Curve 2, inhibition by hexahomoserine, 1×10^{-2} M; Curve 3, uninhibited incorporation. Incorporation expressed as in Fig. 1.

FIG. 3. Double reciprocal plot showing noncompetitive inhibition by δ-hydroxylysine of lysine incorporation into bone marrow protein. Curve 1, inhibition by δ-hydroxylysine, 1×10^{-2} M; Curve 2, uninhibited incorporation. Incorporation expressed as in Fig. 1.

Effect of Erythropoiesis on P^{32} Uptake by Deoxyribonucleic Acid of Spleen in the Rat.* (24580)

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Erythropoiesis has been a subject that has commanded great interst for many years. A number of parameters have become "classical" methods for measuring erythropoiesis. Some of these parameters are: red cell counts, packed red cell volume, circulating hemoglobin per cent, and reticulocyte per cent. More recently, various radioactive isotopes have been used to measure erythropoiesis, among these are C^{14} and Fe^{59}. The method for measuring increased erythropoiesis to be described utilizes differences in radioactive phosphorus (P^{32}) uptake by the deoxyribonucleic acid (DNA) fraction of the rat spleen(1) of erythropoietically stimulated and non-stimulated animals. Rats were stimulated by standard hemorrhage, anoxic anoxia, and injections of anemic rabbit or hypoxic rabbit plasma.

Chemical methods. The method used for isolating DNA from rat spleen was a modification of the Schmidt-Tannhauser procedure (2). Experimental and control animals were injected intraperitoneally with 2 μc P^{32} per 100 g of body weight. Exactly 4 hours after injection the animals were sacrificed and the spleens removed. Three or 4 spleens were pooled, immersed in 3 or 4 volumes of 10% NaCl, and homogenized for one minute in a micro Waring Blendor. The homogenates were heated in a water bath at 85°C for 4 hours. After heating, the homogenates were filtered through medium sintered glass with the aid of a small amount of Celite to remove splenic residue. Three volumes of 95% ethanol were added to the filtrate. The resulting suspension was centrifuged and the supernatant discarded. Fifteen ml of 1 *N* KOH were added to dissolve the precipitate. The solution was incubated at 37°C for 18 hours. After incubation 1.5 ml of 6 *N* HCl and 3.0 ml of 10% trichloracetic acid were added. This step precipitates the DNA and separates it from the RNA. The DNA precipitate was washed with 95% ethanol and ethyl ether. 0.1 *N* NaOH was added to the dried material and a small amount of heat applied to insure solution. One ml of this solution was placed on a planchet and dried for gas-flow counting. A 0.2 ml aliquot was taken for total phosphorus determination by the method of Gomori(3). *Standard hemorrhage.* Male Wistar strain rats weighing 180-200 g were bled 2% of the body weight by cardiac puncture under light ether anesthesia. Prior to the standard hemorrhage, hematocrit and reticulocyte percentages were determined to evaluate the hematologic condition of the rat. Only rats within normal limits were used. At one, 2, 3, 4, 5 and 8 days after hemorrhage, the hematocrit and reticulocyte percentages were determined. Immediately after sampling 2 μc of P^{32} were injected and 4 hours later the rats were sacrificed for splenic DNA isolation as described above. *Anoxic anoxia.* Male rats weighing 180-200 g were placed in a low pressure chamber for 8 hours per day from zero to 4 days at an ambient pressure of 260 mm Hg. Rat pellets and water were supplied *ad lib*. At the designated intervals blood samples were obtained, the animals were sacrificed, and splenic DNA isolated. *Production of anemic rabbit plasma.* Male New Zealand rabbits were made severely anemic by daily injections of phenylhydrazine hydrochloride. After 5 to 7 days of repeated injections, the hematocrit percentage was 8 to 12%. At this time the animals were bled out by cardiac puncture. The plasma from the anemic rabbits was frozen until used. *Production of anoxic rabbit plasma.* Male New Zealand rabbits were placed in a low pressure chamber for 8 hours for 4 days at an ambient pressure of 260 mm Hg. At the end of the fourth day the animals were bled out by cardiac puncture. The plasma from the anoxic rabbits was frozen until used. Male Wistar strain rats weighing 180-200 g were the receptor animals of the "anemic" and "anoxic plasma."

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* Presented at meeting of Am. Physiol. Soc., Sept. 6, 1955.

TABLE I. Effect of Hemorrhage on DNAP of Spleen in 7 Experiments, 3 Rats/Exp.

Time between hemorrhage and sacrifice (days)	Hematocrit, %	Reticulocyte, %	P^{32} uptake* by DNA of spleen
	at time of sacrifice		
0	50.0	.9	152
1	35.3	4.6	426
2	40.7	4.3	520
3	38.3	5.2	936
4	40.7	9.0	580
5	42.0	5.7	349
8	46.2	.9	236

* Expressed as cpm/0.1 mg P.

The animals were injected intraperitoneally with 2 or 4 ml of plasma or saline. Sixteen to 18 hours later 2 μc P^{32} per 100 g body weight was administered by intraperitoneal injection. Four hours after P^{32} injection the animals were sacrificed. The spleens of 3 or 4 rats were pooled for DNA isolation as previously described.

Results. Standard Hemorrhage. The P^{32} uptake by the DNA of the spleen increased to a maximum 3 days after a standard hemorrhage, Table I. Thereafter the values decreased toward normal through the eighth day after hemorrhage. Reticulocyte percentage attained maximum values by the fourth day after hemorrhage, then decreased to normal. Hematocrit percentage was lowest on the first day after hemorrhage, gradually increasing toward normal through the eighth day post-hemorrhage.

Anoxic anoxia. P^{32} uptake of splenic DNA of rats exposed to varying periods of anoxic anoxia is greater than control values (Table II). However, variation is considerable and follows no general pattern. The reticulocyte per cent and hematocrit per cent increases with increase in duration of exposure to anoxia. The latter results agree with previous observations of others and in this laboratory.

TABLE II. Effect of Anoxia on DNAP of Spleen, 3 Rats/Experiment.

Days exposure in low pressure chamber	Hematocrit, %	Reticulocyte, %	P^{32} uptake by DNA of spleen
	at time of sacrifice		
0	45.3	1.1	328
1	44.2	1.8	1180
2	48.0	2.7	600
3	50.2	3.7	1125
4	52.0	8.1	912

Anemia rabbit plasma. Table III compares P^{32} uptake by splenic DNA of normal rats injected with 4 ml of anemic rabbit plasma or saline. Of the 10 individual experiments with 3 or 4 rats per group, with one exception, P^{32} uptake values of the anemic rabbit plasma injected rats are 2 to 5 times greater than the saline injected controls. This difference is statistically significant, ($p < 0.01$).

Hypoxic rabbit plasma. Plasma from anoxic rabbits was injected into normal rats, followed by P^{32} as previously described. The data (Table IV) suggest erythropoietic stimulation in rats following a 4 ml injection of hypoxic rabbit plasma. Erythropoietic stimulation of anoxic rabbit plasma does not appear as great as that following injection of anemic rabbit plasma. This is in general agreement with other observations in this laboratory using other methods.

TABLE III. Effect of Anemic Rabbit Plasma on P^{32} Uptake by Spleen DNA, in 10 Exp.

Saline	Anemic rabbit plasma
cpm/0.1 mg P	
262	446
240	710
210	446
216	369
158	445
158	588
322	262
280	469
308	284
90	140

$p < .01$.

TABLE IV. Effect of Anoxic Rabbit Plasma on P^{32} Uptake by Spleen DNA, in 5 Exp.

Saline	Anoxic rabbit plasma
cpm/0.1 mg P	
353	614
210	945
87	174
87	102
87	121

Discussion. Current opinions in the field of cell division generally agree that when a cell divides mitotically, there is a doubling of DNA. When an animal is erythropoietically stimulated (*i.e.* hemorrhage, anoxic anoxia) a greater number of cells divide in the erythropoietic organs to fulfill the demand for new cells created by the stimulus; therefore, quantity of DNA is increased. Since the spleen is an erythropoietic organ under certain conditions, it was reasonable to assume an increase in splenic DNA in stimulated animals. This increase was measured by the P^{32} uptake by the DNA of the spleen after standard hemorrhage or anoxic anoxia. Carnot(4) first postulated a humoral mechanism for regulation of erythropoiesis. It is generally accepted that in the plasma of erythropoietically stimulated animals there is a factor that will increase red cell formation in normal animals. Using a different method here described, the presence of such a factor in the plasma of phenylhydrazine anemic or hypoxic rabbits has been confirmed.

Summary. 1. A method for evaluating erythropoietic stimulation has been described. 2. Following a standard hemorrhage there is a 2- to 5-fold increase in P^{32} uptake by splenic DNA. 3. Exposure to anoxic anoxia increases P^{32} uptake by the DNA of spleen. 4. Injection of anemic rabbit or hypoxic rabbit plasma increased P^{32} uptake by splenic DNA in normal receptor rats.

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Relation Between Platelet Integrity and Sulfhydryl Groups.* (24581)

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During the last few years platelets have been shown to contain a number of "factors" which are concerned with different phases of clotting mechanism(1). Since it is known that, in formation of thrombin, platelets together with their plasma cofactors produce a powerful intrinsic thromboplastin, it has been postulated that intravascular coagulation may be initiated once appreciable amounts of certain materials are derived from platelets(2). Whether or not release of platelet factors requires actual breakdown of platelets or only changes in their "membrane" permeability characteristics is not as yet known. What is known is that retraction of the fibrin gel requires presence of intact platelets(3) whereas antiheparin and antifibrinolytic activities of platelets are enhanced markedly as result of lysis(4,5). Furthermore, bleeding tendency associated with thrombocytopathy A is related to functional platelet abnormality resulting in increased resistance to disintegration(6). It would thus appear that variations in rate of normal platelet breakdown may have important bearing on several aspects of coagulation mechanism. This paper describes experimental findings suggesting a relationship between platelet integrity and platelet sulfhydryl groups.

Methods and materials. Platelet suspensions were prepared from fresh human blood collected with versene (ethylenediamine tetraacetate Na_2-salt) according to the method of Minor and Burnett(7). Platelets were washed 3 times in physiological saline containing 0.1% versene and 2% Triton WR-1339 (Rohm & Haas), the latter substance used to facilitate resuspension following centrifugation. They were finally suspended in physiological saline containing 2% Triton WR-1339 to give "standard suspension" containing approximately 2.5 x 10^6 platelets/mm^3. Sulfhydryl inhibitors, studied for effects on platelet stability, were dissolved in de-ionized distilled water, adjusted to pH 6.5, and added to "standard platelet suspension" in small volumes to preserve isotonicity. Reduced glutathione and cysteine ethyl ester were dissolved in de-ionized distilled water immediately prior to use, and adjusted to pH

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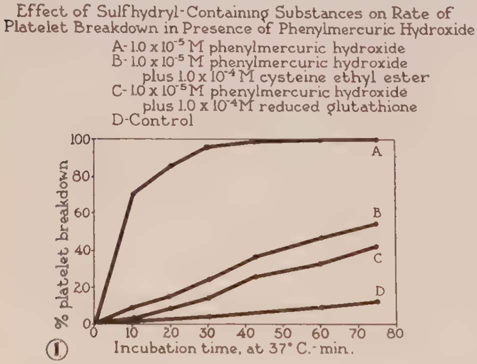
* Supported by Office of Surgeon General, Dept. of Army.

6.5. Samples in which equal volume of physiological saline replaced the test substances served as control. The extent of platelet breakdown was determined by direct count according to the method of Rees and Ecker (8). All counts were carried out in duplicate, the standard error of method being ± 11%. All experiments were carried out at least in triplicate and the results reported are averages. All glassware in the collection of blood, in preparation of platelet concentrates and in experiments was silicone-coated (G. E. Dri Film #9977). Metal surfaces were treated with Arquad(9). Reduced glutathione, N-ethyl maleimide and cysteine ethyl ester were obtained from Schwarz Lab., Na-iodoacetate and alloxan were Eastman Kodak products, p-chloromercuribenzoic acid and iodosobenzoic acid were obtained from Sigma Chemical Co., Salyrgan from Winthrop-Stearns, and phenyl mercuric hydroxide from Matheson, Coleman and Bell. All other chemicals used were of standard reagent grade.

Results. Freshly prepared human platelets were incubated with phenyl mercuric hydroxide, a mercaptide-forming agent known to inhibit many SH-dependent enzymes. Platelet destruction occurred rapidly, although in presence of SH-containing substances, such as reduced glutathione or cysteine ethyl ester, its rate was markedly decreased (Fig. 1). Findings similar to these have been reported for sheep erythrocytes by Benesch and Benesch (10) and for human erythrocytes by Sheets, Hamilton and DeGowin(11).



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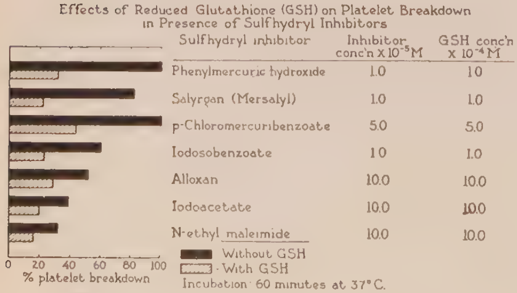

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FIG. 1 and 2.

Results obtained with various other sulfhydryl inhibitors, both in presence and absence of reduced glutathione, are presented in Fig. 2. Regardless of type of reaction which these substances undergo with sulfhydryl groups, an increased rate of platelet breakdown is observed not only with mercaptide-forming agents, but also with alkylating substances such as iodoacetate and sulfhydryl-oxidizing agents such as iodosobenzoate. In all cases studied the effects observed were highly sensitive to concentration and could always be decreased in extent by suitable concentrations of SH-containing substances such as cysteine or reduced glutathione. The results seem to point towards a relationship between platelet sulfhydryl groups on one hand and the maintenance of platelet integrity on the other.

Discussion. Of particular interest are the results obtained with N-ethyl maleimide. This substance which is highly specific in its ability to react with sulfhydryl groups, brought about a significant breakdown of platelets in concentration of 10^{-4} M. On the other hand, it has been reported that the same material in concentration of 5 x 10^{-4} M does not hemolyze human red cells, although other sulfhydryl reagents do(12). An explanation for the difference between these 2 sets of observations is not immediately apparent, although it may be suggested that interstices of the platelet membrane are larger than those of the red cell, thereby allowing access of N-ethyl maleimide to the critical SH-groups of the intact platelet.

It has been previously reported that whole platelets contain a number of active dehydrogenase systems(13,14) and that activities of some of these enzymes can be materially increased by sulfhydryl-containing substances (13). As it is becoming increasingly evident that platelets do carry out various metabolic activities, one may wonder about the interrelationship between their rate of metabolism and their ability to maintain their structural integrity. Is a normal rate of metabolism a prerequisite for an intact platelet structure or is the maintenance of an intact structure dependent on normal rate of metabolism? At present there is no clear-cut answer to these questions although data on effects of various other types of enzyme inhibitors on platelet stability indicate that it is more likely that platelet structure is dependent on platelet metabolism (unpublished). Thus, it is suggested, although by no means proved, that increased rate of platelet breakdown observed in presence of SH-inhibitors may be brought about by effects of these substances on a variety of SH-dependent enzyme systems essential to normal platelet metabolism.

Summary. 1. Incubation of freshly prepared human platelets with a variety of sulfhydryl inhibitors results in increased rate of platelet breakdown. 2. In all cases studied the effects of inhibitors can be decreased in extent by suitable concentrations of SH-containing substances such as cysteine or reduced glutathione. 3. Results obtained suggest a direct relationship between platelet sulfhydryl groups and platelet structure. The possible link of this relationship to platelet metabolism is briefly discussed.

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Cytopathogenic Effect Produced by Polyoma Virus in Cultures of Milk-Adapted Murine Lymphoma Cells (Strain P388 D_1).* (24582)

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Production of pleomorphic tumors of the parotid glands of mice by inoculation of newborn animals with cell-free filtrates prepared from mouse neoplasms has been described both by Gross(1) and by Stewart,(2,3) and has been confirmed in several laboratories(4,5,6). In addition to parotid gland tumors, the inoculated mice have often developed tumors in other salivary glands, mammary glands, the thymus gland, the subcutaneous tissue and other sites. Recently, Eddy and her associates have reported that preparations of the agent also produce sarcomatous and angiomatous tumors in hamsters(7). Evidence suggesting replication of the agent in monkey kidney cell cultures was obtained, but cytopathogenic changes were not observed in most of the infected cultures(8). Subsequently, it was shown that the agent could be propagated in minced mouse embryo cultures, and cytopathogenic changes were observed with some preparations(9). Recently, Eddy and her as-

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* We appreciate the technical assistance of Mr. Arleigh Green.

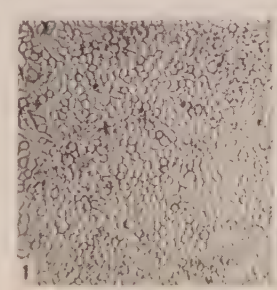
sociates have found that the agent consistently produces cytopathogenic effects in mouse embryo cultures, and that this activity is correlated with induction of tumors in hamsters (10). Hemagglutination of guinea pig erythrocytes by the agent has also been described, and hemagglutination inhibiting antibody has been found in sera of animals inoculated with infected tissue culture fluids(11). Eddy and her associates have suggested that the agent they have studied be called SE Polyoma Virus (10,11). This paper describes a cytopathogenic effect produced by polyoma virus in cultures of a cell strain (P388 D_1) derived from a murine malignant lymphoma(12). The virus produces cellular destruction in 2 to 6 days with undiluted inocula, and virus pools prepared in P388 D_1 cell cultures have titers as high as $10^{7.5}TCID_{50}$ in 0.25 ml. The cell strain has been adapted to growth in a serum-free medium containing 20% autoclaved non-fat milk, and, therefore, the cell cultures are free of serum antibodies(13). Cultures can be inoculated with virus while in this serum-free growth medium and can be observed for long periods of time, since the control cultures do not degenerate, as usually occurs in other tissue culture virus-host cell systems in low serum concentrations or in serum-free maintenance media.

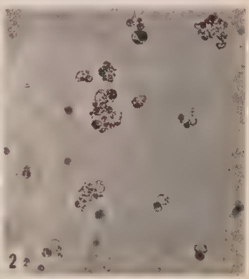
Materials and methods. Viruses. The first preparation of polyoma virus studied was obtained from Dr. Bernice Eddy and Dr. Sarah Stewart and was designated by them as "strain 210 S. E. Polyoma." It consisted of tissue culture fluid from the 35th passage of the agent in mouse embryo tissue culture and had been through 2 passages in mice and 2 passages in hamsters. In the animals, tumors had been produced as has been described by Stewart and Eddy(3,7,8,9,10). A second preparation of virus was obtained from Dr. C. J. Dawe and Dr. L. W. Law of the National Cancer Institute. This preparation was a cell-free Selas 03 filtrate of an extract of a parotid gland tumor from a $C_3H/_{Bi}$ mouse. On the day of birth this mouse had received material from a short-term tissue culture of a thymic tumor. The thymic tumor, in turn, had appeared in a $(C_3H_{fx}AKR)_{F1}$ mouse that had received, on the day of birth, material from a short-term culture of leukemic tissue from a $C_3H/_{Bi}$ mouse. The leukemia in this animal was in its eleventh serial mouse passage, and had originated in a mouse of the "Passage A" series of the experiments of Dr. Ludwik Gross. The derivation of this strain of virus and some of its biologic properties will be described in greater detail by Dawe, Law and Dunn. *Cell cultures.* The cell strain (P388 D_1) used in these studies was originally isolated by Dawe and Potter, from the 49th mouse passage of a methylcholanthrene-induced lymphoid neoplasm (P388) of a DBA/2 mouse(12). They described a morphologic alteration in tumors derived from cultivated cells as compared with the serially mouse-passaged parent tumor. The culture-derived tumors resembled reticulum cell sarcomas, and the altered cells showed marked phagocytic activity *in vitro*. The cell strain was grown in a medium containing 40% human serum up to the time we received it. To eliminate serum antibodies and serum inhibitors, we have adapted this cell strain to growth in a serum-free medium containing 20% autoclaved non-fat milk and 80% medium #199. Adaptation of these cells to the serum-free medium, preparation of the medium and methods of growing the milk-adapted cells have been described(13). In the virus experiments, full grown tube cultures of milk-adapted P388 cells containing approximately 1,500,000 cells and 2 ml of the milk medium have been inoculated with 0.25 to 0.5 ml of virus preparation. After inoculation, the cultures continue to lower pH of medium and are fed every 2 to 3 days by removing 1 cc of medium and replacing it with 1 cc of fresh medium. When the cytopathogenic effect is evident, the entire culture including both cells and supernatant fluid is frozen at –20 degrees and stored for future passage in tissue culture or in animals. Since the inoculated cultures are in serum-free growth medium, it would be possible to observe tubes in virus titrations for very long periods of time if they were fed with new medium at appropriate intervals. We have found, however, that even high dilutions of virus produce a cytopathogenic effect within 21 days, and we have usually terminated titration experiments after 30 days. It is possible, though, that some of the tubes inoculated with high dilutions of a virus pool which did not show cytopathogenic effect at 30 days might have done so if observed for longer periods of time. *Animal inoculations*. Pregnant golden hamsters were obtained from the animal colony of the Nat. Inst. of Health. Newborn hamsters, on day of birth or one day after, were inoculated with 0.05 cc of polyoma virus grown in milk-adapted P388 D_1 cells. The preparations were not filtered and, thus, contained cellular debris and milk medium as well as the virus. The newborn hamsters were inoculated under the skin of the back and were observed daily. Complete necropsies were performed on all animals found dead.

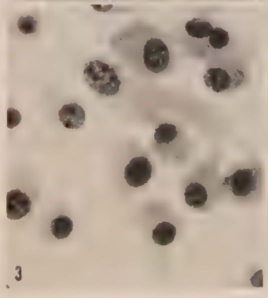
Results. Two tube cultures of milk-adapted P388 D_1 cells were inoculated with 0.5 cc of undiluted "strain 210 S. E. Polyoma" obtained from Drs. Eddy and Stewart. Seven days after inoculation, a cytopathogenic effect was observed in the inoculated cultures. Large numbers of cells had detached from the glass surface leaving only scattered clusters of pyknotic and fragmented cells. Supernatant fluid from these cultures was passaged to new tube cultures of milk-adapted P388 D_1 cells and a similar cytopathogenic effect was noted 7 to 9 days after inoculation. The virus has now been carried through 6 consecutive passages with a total effective dilution of 10^{-14}. In the sixth passage, cytopathogenic effect was observed 4 days after inoculation. Titration of a pool of fourth passage material by serial 10-fold dilutions in milk-adapted P388 D_1 cells has shown a titer of $10^{5.5}$ $TCID_{50}$ in 0.25 ml.

After observation of a cytopathogenic effect in cultures of milk-adapted P388 cells with "strain 210 S. E. Polyoma" virus, the cell-free parotid gland tumor filtrate provided by Dawe and Law was studied in the same cell culture system. Fifteen days after inoculation of the P388 D_1 cultures with the parotid gland tumor filtrate, some questionable cytopathogenic effect was noted in the inoculated tubes and supernatant fluids from these tubes were passaged to new tubes of milk-adapted P388 D_1 cells. Six days after inoculation, cytopathogenic effect was definite in the tubes of this second passage. The cytopathogenic effect was similar to that seen in tubes inoculated with the "strain 210 S. E. Polyoma." Subsequently, this agent has been carried through 11 consecutive passages with a total effective dilution of 10^{-21}. A pool of eleventh passage material produces a cytopathogenic effect in 2 days with undiluted inocula. Titration of this pool by serial 10-fold dilutions in milk-adapted P388 D_1 cells shows a titer of $10^{7.5}$ $TCID_{50}$ in 0.25 ml. Inoculation of tube cultures with 5 $TCID_{50}$ of agent caused cytopathogenic effects in 15 to 21 days. A tube culture inoculated with 5 $TCID_{50}$ and incubated until the cytopathogenic effect was evident was found to contain more than 8,000,000 $TCID_{50}$.

Cytopathology. Morphologically, cultures of P388 D_1 cells differ from HeLa cells and the other epithelial-like cell strains commonly used in virus studies. Instead of the confluent cell sheet seen in control cultures of HeLa, KB and similar cell strains, fully populated cultures of P388 D_1 contain large numbers of closely approximated, round, oval and somewhat elongated cells (Fig. 1). Scattered







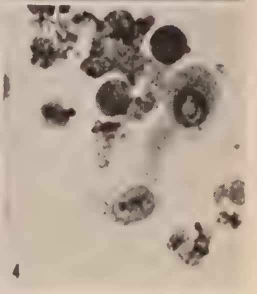


FIG. 1. Control culture of milk-adapted P338 D_1 cells. Unstained, ×70.

FIG. 2. Cytopathogenic effect produced by polyoma virus in culture of milk-adapted P388 D_1 cells. Unstained, ×70.

FIG. 3. Control culture of milk-adapted P388 D_1 cells. Papanicolaou stain, ×415.

FIG. 4. Cytopathogenic effect produced by polyoma virus in culture of milk-adapted P388 D_1 cells. Papanicolaou stain, ×415.

clumps of cells which have become heaped upon one another during growth are seen, and multinucleated giant cells are found in variable numbers.

The earliest evidence of a cytopathogenic effect in cultures of milk-adapted P388 D_1 cells inoculated with polyoma virus is the appearance of scattered small collections of necrotic cells. These cells are shrunken, distorted and more refractile than normal P388 D_1 cells. After these necrotic foci are observed, the cytopathogenic effect usually proceeds rapidly and, in 24 hours, only scattered clusters of necrotic cells remain adherent to the dependent surface of the tube (Fig. 2). Ultimately, all the cells are detached from the surface of the tube. It has not been possible to regrow a cell population in an inoculated culture by feeding fresh medium after the cytopathogenic effect has been observed.

Cultures inoculated with "strain 210 S. E. Polyoma" and cultures inoculated with the agent obtained from Dawe and Law have shown identical cytopathology.

Coverslip cultures inoculated with polyoma virus have been fixed and stained in various stages of the cytopathologic process. In cultures in which the cytopathology has been permitted to proceed to a point where most of the cells have become detached from the coverslip, scattered clumps of cytoplasmic and nuclear debris remain, with occasional clusters of cells with karyorrhexis (Fig. 4). Cells with nuclear enlargement, margination of chromatin along the nuclear membranes, and central clear areas in the nuclei are also seen. Small clumps of eosinophilic material can be seen in some of the clear areas in the nuclei in coverslips stained with H. and E. In the cytoplasm of some cells of cultures with cytopathogenic effect, phagocytized cellular detritus and necrotic cells are seen.

Animal Inoculations. Newborn hamsters have been used to evaluate the oncogenic properties of polyoma virus prepared in cultures of milk-adapted P388 D_1 cells. Four litters of hamsters were inoculated with 6th passage material derived from the mouse parotid gland tumor filtrate received from Dawe and Law. The total effective dilution of the parotid gland material in this 6th passage was approximately 10^{-20}. Forty-two animals were inoculated and all were dead within 40 days of inoculation. Since many of the dead animals were lost due to cannibalism by cage mates, only 19 of the 42 were examined at necropsy. All of these animals had multiple sarcomatous and angiomatous lesions involving heart, kidneys, liver and other sites, which grossly and microscopically are similar to the lesions described by Eddy and her associates in hamsters inoculated with polyoma virus(7). Inoculation of newborn hamsters with "strain 210 S. E. Polyoma" passaged three times in milk-adapted P388 D_1 cells has also resulted in production of similar lesions in the treated animals.

The culture fluids used for animal inoculation were not cell-free filtrates and did contain necrotic P388 D_1 cells which had been frozen and thawed once prior to injection into the hamsters. None of the tumors seen in the hamsters resembled the P388 D_1 malignant lymphoma histologically and there is no reason to suspect that the sarcomatous and angiomatous tumors we have observed represent transplantation of P388 D_1 cells to the hamsters. A control litter of 11 newborn hamsters has been inoculated with a suspension of P388 D_1 cells without polyoma virus after one cycle of freezing and thawing and no tumors have been observed in these animals 3 months after inoculation.

Discussion. The virus-host cell system of polyoma virus in milk-adapted P388 D_1 cells here described is of interest for several reasons. Rapid production of a cytopathogenic effect makes the system suitable for titrations and growth studies of the agent. Since the cell strain grows in serum-free medium, it is possible to study infection during growth of the cell population in the absence of serum antibody and serum viral inhibitors. The possible presence of inhibitors of polyoma virus in the milk medium, however, has not been determined.

Only a few reports have been found in which viruses were studied in cultures of cells of the histiocyte-macrophage-reticulum cell family, and in none of these was a generalized cytopathogenic effect noted(14,15,16). Cells of the P388 D_1 culture strain morphologically resemble atypical histiocytes or reticulum cells, and retain their phagocytic properties as well as their ability to grow into ascites neoplasms when inoculated intraperitoneally into DBA/2 mice. The fact that polyoma virus causes cytopathogenic effects in the cells of such cultures suggests that P388 D_1 cells might be of interest in studies of other viruses.

Summary. A cytopathogenic effect has been observed in cultures of murine malignant lymphoma cells (strain P388 D_1) inoculated with polyoma virus. There is evidence of viral multiplication in these cell cultures and the virus retains its ability to produce tumors when inoculated into newborn hamsters. The virus-host cell system of polyoma virus and milk-adapted P388 D_1 cells seems to be a suitable one for studies of this oncogenic agent.

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Serum Proteins in Aminopterin Treated Rats. (24583)

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Importance of folic acid in metabolism of proteins and nucleoproteins has been recently reported. That folic acid is involved in purine and creatine metabolism, has been shown by Dinning and Day in monkeys(1) and Totter *et al.*(2) in chicks. A decrease in incorporation of C^{14} formate in nucleic acid(3) and in liver and visceral protein(4) in folic acid-deficient rats implicates this vitamin in biosynthesis of proteins and purines. Bakerman and coworkers(5,6) observed an increase in excretion of aminoacids, mainly glutamic acid, in folic acid deficiency, while Ricceri (7) observed a fall in excretion of uric acid and allantoin. Similar studies on blood have been reported by Tentori and Vivaldi(8). These observations indicate that folic acid is intimately related to protein metabolism. The present investigation was undertaken to study whether folic acid deficiency would influence the level of total serum protein or any of its electrophoretic components in rats. Fibrinogen content of plasma of folic acid deficient and pair-fed normal controls was also studied.

Methods. Adult male rats, weighing 180-240 g were fed mineralized whole milk with 2 drops of a concentrate of Vit. A and D, twice a week. Animals which grew well were paired and housed in individual metabolism cages. Folic acid deficiency was produced in one of the pairs by intraperitoneal injection of aminopterin (25 γ/day). The other rat of the pair served as control and was fed the same amount of milk as its deficient counterpart. Within 10 days of aminopterin injection rats showed the typical deficiency syndrome. Porphyrin appeared around eyes and

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* National Research Fellow, Govt. of India.

TABLE I. Hematological Picture in Rats.

	Hemoglobin, g/100 ml (12)	Hematocrit value, % (5)	Leucocytes, mm^3 (4) (× 1000)
Normal	15.51 ± .22*	47 ± .5	6.33 ± .37
Aminopterin treated	10.42 ± .27	30 ±2.0	.60 ± .14

* Mean ± S.E.
Figures in parentheses indicate No. of animals.

TABLE II. Plasma Fibrinogen Values in Rats, g/100 ml.

	Plasma fibrinogen (6)
Normal	.21 ± .02
Aminopterin treated	.55 ± .06

nose, diarrhoea was evident, the animals became inactive with loss of appetite and significant weight loss. In the evening, food was withdrawn from cages and the next morning both deficient and pair-fed normal animals were anaesthetized with pentobarbital sodium. The left carotid was exposed and a fine needle tied into it. The needle was connected to a 15 cc rubber stoppered centrifuge tube. With the help of slight suction, the blood immediately collected in the tube which was allowed to clot for serum. A small quantity of blood was taken in an oxalated tube. From this, hematological findings and samples were taken and the rest centrifuged without delay for plasma. Vitamin deficiency was established by leucocyte count and determinations of hemoglobin and packed cell volume. Total nitrogen and non-protein nitrogen of serum and plasma fibrinogen were determined by methods described previously(11). Serum proteins were separated by paper electrophoresis. 0.01 cc undiluted serum was used and electrophoresis continued for 16 hrs with a current of 4 mA and 200 volts in barbiturate-barbituric acid buffer of pH 8.6 and ionic strength 0.05. Staining of paper strips, determination of optimum density curves of the coloured zones by densitometry and calculation of amounts of different fractions of serum proteins were the same as described previously(11).

Results. Results are given in Tables I, II and III. A low hemoglobin content, diminution in number of white blood corpuscles and a low hematocrit value were noted in aminopterin-treated rats (Table I). The serum protein pattern of these animals was characterized by lowering of albumin and γ-globulin and increase in α 2-globulin values. These values have significance at the p 0.01 level. There was no significant change in α_1 and β-globulin fractions. There was a decrease in total serum protein, but a significant increase in fibrinogen content in aminopterin induced folic acid deficient animals when compared to normal controls.

Discussion. Folic acid is reportedly involved in nucleoprotein synthesis(3) and ribonucleoprotein is probably related to protein synthesis(12). It is possible that in animals deficient in folic acid formation of nucleoprotein and hence protein synthesis is retarded(4). This is a possible explanation of our finding of decreased serum protein and albumin. Since both fibrinogen and albumin are made in the liver the rise in fibrinogen indicates that aminopterin interference with liver function is not a simple depression of protein synthesis(13,14). The present findings with aminopterin treatment are similar to those with ascorbic acid deficiency(9). This may be because metabolism of folic acid and ascorbic acid are closely interrelated(15), or because this is the usual response of the liver to injury (Ed.).

The electrophoretic pattern of serum globulins with aminopterin treatment is not similar to that of combined folic acid and Vit. B_{12} deficiency reported by Mulgaonkar and Sreeni-

TABLE III. Different Fractions of Serum Proteins in Rats (%).

Rats	Albumin	Globulins				A/G ratio
		α_1	α_2	β	γ	
Normal (12)	2.06 ± .07	1.20 ± .06	.41 ± .02	1.20 ± .05	1.12 ± .06	.52
% of total protein	34.39	20.03	6.85	20.03	18.70	
Aminopterin treated (12)	.83 ± .05	1.14 ± .08	.84 ± .05	1.18 ± .07	.43 ± .04	.23
% of total protein	18.78	25.79	19.00	26.70	9.73	

vasan(10). Our values with normal rats, however, compared favourably with theirs. In their case a decrease in α_1-globulin with significant increase in β-globulin was observed, whereas under our conditions an increase in α_2-globulin with a decrease in γ-globulin was observed.

Summary. Different fractions of serum proteins were determined by paper electrophoresis in folic acid deficient and pair-fed normal rats. Total serum protein was low and plasma fibrinogen was significantly high in the deficient animals. There was a significant decrease in albumin and γ-globulin, considerable increase in α_2-globulin, with no significant change in α_1 and β-globulin fractions in the serum of folic acid deficient rats.

Aminopterin was kindly supplied by Lederle Lab. (India).

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Exchange of Radioactive Magnesium in the Rat.*† (24584)

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Changes in extracellular concentration of magnesium have been implicated in various pathological states and in mammalian hibernation. The clinical aspects have been reviewed by Flink(1) and Elkinton(2) and the role of magnesium in hibernation by Riedesel(3). The recent availability of a radioactive isotope, Mg^{28}, has provided a tool for investigating the dynamic equilibrium between extracellular magnesium and that in the cells and/or skeleton. The present report concerns the exchange of magnesium between plasma and soft tissues in the rat.

Methods. Mg^{28} is an isotope emitting both gamma and beta radiation with a half-life of 21.3 hours. It decays to Al^{28} which also emits gamma and beta radiation with a half-life of 2.3 minutes, and this daughter decays to stable Si^{28}. The isotope, with Mg^{24} carrier, was obtained from the Brookhaven National Laboratory and was prepared to yield a solution containing 150 meq/l magnesium. Twenty-six male rats ranging in weight from 290 to 325 g were each injected with about 0.5 ml of this solution intraperitoneally. The injections were made from a tuberculin syringe which was weighed on an analytical balance before and after each injec-

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tion. At intervals after injection each rat was anesthetized and exsanguinated through a needle in the abdominal aorta. One ml samples of plasma were taken for radioactivity determination and chemical analysis for magnesium. The red cells were washed twice in ice-cold saline and radioactivity of 1 ml of packed cells was counted. The other tissues sampled included liver, left ventricular myocardium, kidney, brain (whole cerebral hemispheres), erythrocytes, testis, diaphragm, and gastrocnemius. In 14 rats the radioactivities of the erythrocytes were determined but not those of the brain, and vice versa in the other 12. A similar experiment was conducted with 11 immature male rats weighing 150 ± 10 g. The tissues sampled were as above, including brain but excluding erythrocytes. The uptake of radioactive magnesium by the rat diaphragm *in vitro* was also studied. Hemidiaphragms from rats weighing 150 ± 10 g were dissected and incubated for 50 minutes in a solution containing radioactive magnesium. The medium was a Krebs-Ringer, bicarbonate-CO_2 buffered solution containing 100 mg % glucose and 200 units/l insulin. Each hemidiaphragm was weighed and placed in a separate 25 ml Erlenmeyer flask with 3 ml of the nutrient solution and shaken at 180/min. in a water bath at 37°. They were removed at intervals and their radioactivities measured. Incubation time of each hemidiaphragm was different from that of the other hemidiaphragm from the same animal. Finally, uptake of radioactive magnesium by stimulated muscles was compared with that of unstimulated muscles. Two male rats weighing about 300 g were anesthetized with sodium pentobarbital and the sciatic nerve exposed in each thigh. A stimulator electrode was placed under each nerve but only one side was actually stimulated. The stimulations were from a Harvard stimulator at 2 per second. The rats were killed after 27 and 52 minutes, respectively, and radioactivities of the gastrocnemii from the 2 sides were compared. Plasma and tissue samples were placed in a well-type scintillation counter and counted for 2 minutes or until 2000 counts had been recorded. After suitable corrections for decay and the injected dose per kg body weight, specific activities of plasma and tissue magnesium were calculated. The specific activity of the magnesium in each tissue was divided by that of the plasma magnesium at time of sampling. This gives "relative specific activity" (r.s.a.), which indicates the degree of equilibration of the magnesium in plasma and tissue. A value of 1 indicates complete equilibration. The plasma samples were analyzed for magnesium by the method of Orange and Rhein(4). This is essentially a colorimetric determination of a magnesium-Titan Yellow complex in the presence of a colloidal material, in this case polyvinyl alcohol. For the other tissues mean values from the literature for the absolute magnesium content were used(5). Calculations were made using values for each extreme of the range reported in the literature and where no such range was given, it was arbitrarily assumed to be ± 25%. In Fig. 1, b, c, d, and f, the solid line shows increase of r. s.a., using the mean value for magnesium content of the tissue and the broken lines show the 2 ranges.

Results. A. *Plasma.* The decay of the standardized specific activity of plasma magnesium in mature rats is shown in Fig. 1 a. There are probably 2 exponential processes. The slope of the slower component was fitted by eye and extrapolated to the y-axis so that value of the slow component could be determined at each experimental interval. These values were subtracted from the experimentally determined activities and the differences plotted semilogarithmically. The slope of this new line, plotted as the broken line in Fig. 1 a, is taken as that of the faster process. It was found to be –0.87, that of the slower component, –0.04. In the immature rats the slopes were –0.93 and –0.07, respectively.

B. *Tissues.* In the exchange of plasma magnesium with that in the cells, tissues seem to fall into 2 classes. In liver, heart, and kidney the exchange is rapid and reaches complete equilibrium in about 3 hours. In brain, testis, red cells and skeletal muscle the exchange is not complete after 7 hours. *Liver, heart, and kidney.* The increase of relative standardized specific activity (r.s.a.), of heart

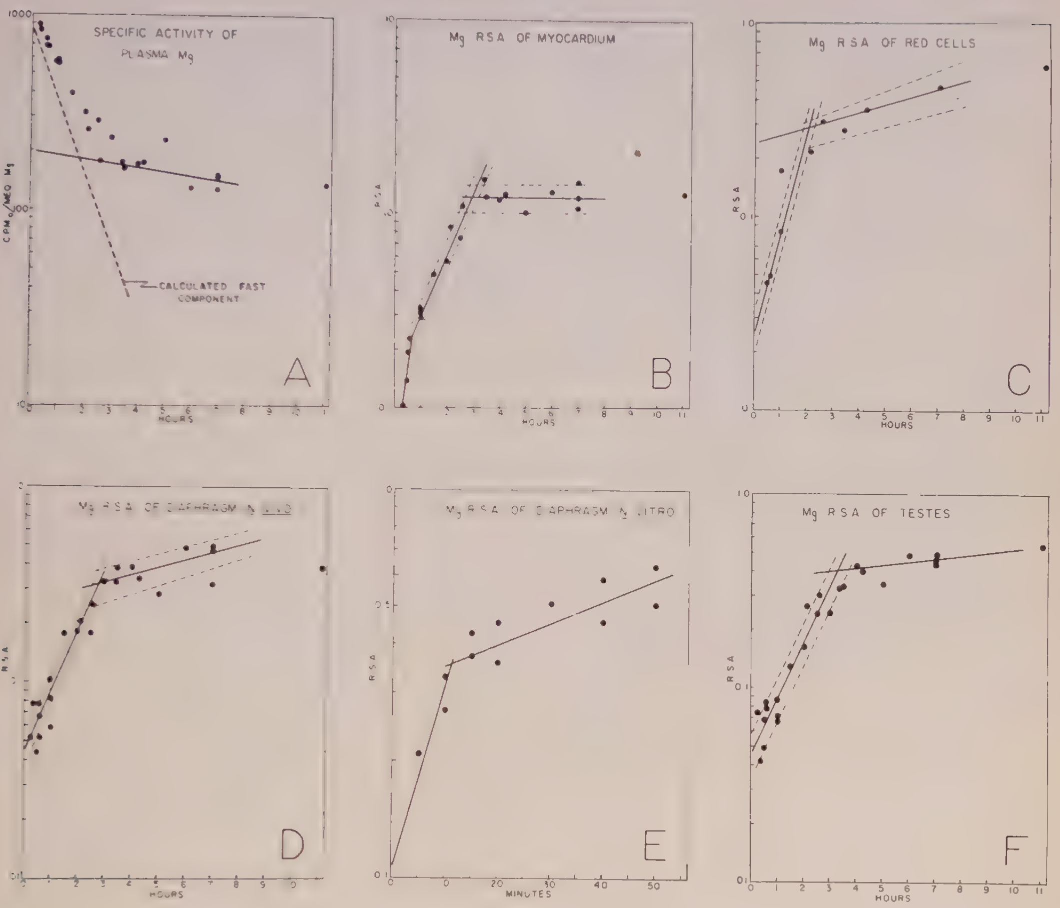

FIG. 1. R.S.A. is relative specific activity. R.S.A. $= \frac{\text{specific activity of tissue Mg}}{\text{specific activity of plasma Mg}}$. Broken lines indicate range of R.S.A. calculated from normal range of Mg content in the appropriate tissue.

muscle in mature rats is shown in Fig. 1 b. The graphs for liver and kidney are very similar to this. In each case there appear to be 2 fast components with an inflection at about one-half hour after injection. Complete equilibration is achieved at about 3 hours. Overall increase before equilibration is reached has a slope of 0.78 which is similar (but with the reverse sign) to that for plasma decay. The comparable value for heart in the immature animals is 0.74. *Red cells.* The exchange of magnesium in red cells is shown in Fig. 1 c. The data do not permit an analysis of the components of the exchange but it is clear that there is some magnesium in rapid exchange with that in the plasma and at least one other magnesium "compartment" in relatively slow exchange. The exchange is only 45% complete after 7 hours and in a single rat sacrificed after 11 hours equilibration was 60% complete.

Skeletal muscle. The increase in the r.s.a. in diaphragm is shown in Fig. 1 d. The scatter of points is greater than in some of the other tissues but an inflection at about 3 hours is apparent. In the gastrocnemius r.s.a. is lower but follows a similar pattern. After 7 hours equilibration is 50% complete in the diaphragm and 20% in the gastrocnemius. The slope of the initial increase in the r.s.a. in diaphragm is 0.75.

Diaphragm in vitro. The initial process in equilibration of the diaphragm *in vivo* is strikingly similar to that of the rat diaphragm

TABLE I. Uptake of Radioactive Magnesium by Stimulated Gastrocnemius.

Duration of stimulation, min.	cpm/kg × 10^{-3} Stimulated	Unstimulated
27	106	44
52	271	169

incubated *in vitro,* shown in Fig. 1 e with a different time scale. Slope of the *in vitro* equilibration is 0.78. The rapid increment of the r.s.a. during the first 10 minutes of incubation represents presumably the exchange of magnesium in the interstitial fluid.

Stimulated gastrocnemius. Table I lists the results of *in vivo* stimulation of the gastrocnemius. In both cases the stimulated muscle has much higher radioactivity than the unstimulated.

Brain and testes. The increase in the r.s.a. of the magnesium in testes is plotted in Fig. 1 f. Equilibration is only 45% complete after 7 hours and a single value at 11 hours shows 55% equilibration. Here again there are 2 components analagous to those in the plasma. The pattern of exchange in the brain is similar but the initial increase is slower and equilibration after 7 hours is 25%. In the immature rats the slope of initial increase in r.s.a. of testes is the same as in the older animals but is continued for longer so that after 7 hours the testes are 70% equilibrated rather than 50%.

Discussion. The use of mean values for magnesium content of the tissues is evidently adequate for interpretation of the radioactivity data. In each case (Figs. 1 b, c, d, and f), the results calculated from the extremes of each range of values are not significantly different from the mean and do not affect the estimate of the time at which equilibration is reached. This is true even of those tissues in which the range is assumed to be as high as ± 25%. Furthermore, measurement of the slope is independent of absolute magnesium content of the tissue.

Following injection of the magnesium isotope, the tissues can become radioactive either by net uptake of magnesium or by exchange of intracellular with extracellular magnesium. The former possibility must be considered. The isotope is not available free of carrier, and so to achieve sufficient radioactivity for detection in the latter phases of the experiment it is necessary to inject more magnesium than is ideal in a tracer experiment. The animals are thus subjected to a slight magnesium load. The very rapid increment of radioactivity of liver, kidney, and heart during the first 30 minutes may be interpreted as a deposition of some of the injected load. As these organs comprise 15% of the body weight and the total load to be distributed amounts to only 0.064 meq, the increase would lie within the normal range of variation in chemical composition even if none were deposited elsewhere.

The most striking aspect of these results is that the organs clearly fall into 2 classes with respect to their uptake of radioactive magnesium. The organs in which the magnesium exchanges rapidly and completely with that of the plasma are the liver, heart, and kidneys. Noonan *et al.*(6) found that in this same group of organs the potassium was in rapid exchange with radioactive potassium in the plasma and that the potassium of brain, testes, and erythrocytes was slow in equilibrating. They also demonstrated, however, that skeletal muscle is intermediate between the 2 groups, and in this respect the results with magnesium are different. Equilibration of muscle magnesium is just as slow as that in the red cells for example, suggesting that the magnesium in muscle is more tightly bound than is the potassium. In skeletal muscle (as in all the "slow" organs) about 20% of the magnesium can exchange readily with the extracellular fluid at a rate with a half-time similar to the main process in the plasma decay. The remainder of the magnesium is relatively inexchangeable.

Within each of the 2 groups of organs there is remarkable similarity in equilibration rates of such disparate tissues as diaphragm and testes, for example. This could be accounted for either by a similarity in permeability of the membranes to magnesium or by the existence of 2 (or more) states of magnesium in the cell, each with its characteristic exchange rate. The latter proposal is more consistent with the observations that there are 2 processes in equilibration in the "slow" organs and that the faster of these processes has the same rate as the single process in the "fast" organs. On the one hand we may consider a "free" magnesium with a turnover time of 1.2 hours and on the other hand, a "bound" magnesium with a turnover time of 25 hours.

In liver and kidney, all of the magnesium would be in the rapidly exchanging state and in skeletal muscle more than half the magnesium would be in the slowly exchanging state. The intimate association of magnesium with the contractile elements of muscle is also consistent with this proposal and it is especially noteworthy that a *stimulated* skeletal muscle shows a more rapid exchange than an unstimulated muscle. The possible storage of magnesium load in the liver, etc., is of interest but by analogy with other electrolytes the skeleton presumably contains the principal reserve of magnesium.

The decrease of specific activity in the plasma of immature rats is slightly faster than in the older rats. This is to be expected even if only from a consideration of their shorter circulation time. In the testes of immature rats initial rate of increase of r.s.a. is the same as in the older animals but it is maintained for longer so that after 7 hours the testes are 70% equilibrated rather than 50%. The implication is that the less mature testes contain more of the rapidly exchangeable magnesium. Otherwise there are no striking differences between mature and immature rats.

Summary. Mature and immature rats were injected with radioacitve magnesium and distribution of the isotope in various tissues was measured. The exchange with plasma magnesium was rapid and complete within 3 hours in liver, kidney, and heart muscle. In brain, testes, erythrocytes and skeletal muscle there was a rapidly exchanging component similar to that in liver, etc., but more than half the magnesium exchanged very slowly. The similarity between functionally disparate tissues in each of the 2 groups suggests that the magnesium is present in 2 or more physiologic states. In one state the turnover time is 1.2 hours and in the other it is 25 hours. The exchange was faster in muscles which were stimulated to contract repeatedly than in resting muscles. In immature rats uptake of radioactive magnesium by the testes was slightly faster than in the mature animals and net decay of plasma activity was faster but there were no other significant differences.

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Effect of Salicylates on Respiration and Phosphorylation in Heart Mitochondria. (24585)

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The observations that salicylate and dinitrophenol significantly increase basal metabolic rate in intact man(1-3) and animal(4-5) have been attributed to the ability of these phenols to uncouple oxidative phosphorylation(6-7). In view of the efficacy of salicylates in treatment of rheumatic fever and rheumatoid arthritis, the cardiovascular effects of salicylate-induced hypermetabolism have received some attention. Tenny and Miller(5) demonstrated in the dog that major cardiovascular adjustments to salicylates

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are increased in cardiac output and widening of arteriovenous oxygen difference. Because there was only a slight increase in pulse rate, it was assumed that the increase in cardiac output was predominantly the result of increase in stroke volume. This has recently been confirmed by Walton and Darby(8) who showed by more direct methods that salicylate injection in the dog produces a prompt increase in contractile force of heart and by Nayler(9) who observed that salicylate and dinitrophenol cause a positive ionotropic response in the isolated toad heart. Finally, studies in euthyroid(10) and myxedematous (11) human subjects have revealed that salicylate-induced hypermetabolism does not cause a significant increment in pulse rate unless toxicity is marked(12). In contrast, Brewster *et al.*(13) demonstrated that thyroid feeding in the dog produces significant increase in heart rate as well as increment in ventricular stroke work. Studies of Tenny and Miller(5) with salicylates and Brewster *et al.*(13) with desiccated thyroid are particularly noteworthy because both used dogs in which the metabolic rate was increased to a similar extent; yet only with thyroid feeding was there a significant increase in pulse rate. Moreover, Alexander and Johnson(11) found that patients with myxedema, restored to normal metabolic rate with triiodothyronine or salicylate, developed an increase in pulse rate and angina pectoris with the former but not with the latter. Thus, since thyroxine, like salicylate and dinitrophenol, is capable of uncoupling oxidative phosphorylation(14-15), the difference in cardiovascular effects of thyroxine and the 2 phenols have been interpreted to indicate that thyroid hormone increases reactivity of the cardiovascular system by some means other than or in addition to increased tissue oxygen consumption(10). It is possible, however, that salicylate is capable of uncoupling oxidative phosphorylation in peripheral tissues but not in the heart. Hence, a study of the effect of salicylate, various analogs of salicylate, and thyroxine on heart mitochondria was undertaken. It has recently been suggested(16) that uncoupling of oxidative phosphorylation is an important characteristic of agents possessing anti-inflammatory properties, it seemed important to confirm Brody's observation(6) that certain salicylate analogs with apparent anti-rheumatic properties do not uncouple oxidative phosphorylation. It was hoped that a study of uncoupling activity of substances chemically related to salicylate would indicate what characteristics were essential for uncoupling by this group of compounds.

Methods. Preparations. Heart muscle mitochondria were prepared from male rats of Sprague-Dawley strain by procedure of Cleland and Slater(17) using a sucrose (0.32 M)-versene (0.001 M) suspending medium at pH 7.5. The prepaartion was made with approximately 5 g cardiac tissue as starting material. Commercially available reagents were used as source for compounds examined. Thiosalicylic acid was recrystallized by sublimation procedures before use. Solutions of the compounds were neutralized with sodium hydroxide and then added to the reaction mixture in approximately 10 μl amounts. Oxygen consumption was measured polarographically with an oxygen electrode (Pt-Ag couple) of the "open type" described by Davies and Brink(18) using the apparatus and assay conditions previously described(19). The reaction mixture (1 ml) contained: 0.32 M sucrose, 9 mM KCl, 18 mM phosphate buffer, 1 mM versene, and 1-2 mg mitochondrial protein at pH 7.5. Other additions during experiments are indicated in the Figures. Time moves from left to right, and a downward deflection of curves indicates oxygen consumption. Numbers on curves refer to calculated rates of oxygen consumption in micromoles oxygen consumed/second/liter. Temperature was 26°C.

Results. Polarographic assay of oxidative phosphorylation and effct of DNP.‡ A control experiment with rat heart mitochondria illustrating the method for assay of respiration and oxidative phosphorylation is shown in Fig. 1. The experiment was begun by addition of aerobic suspending medium to the

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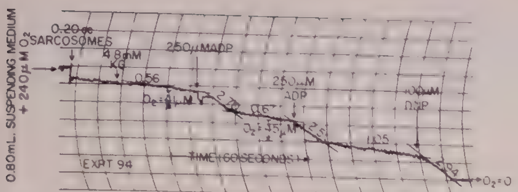
‡ The following abbreviations are used: alpha-ketoglutarate, KG; adenosine diphosphate, ADP; adenosine triphosphate, ATP; 2,4-dinitrophenol, DNP.



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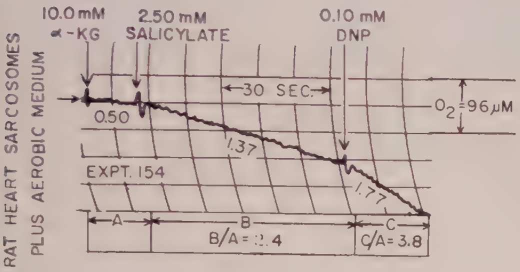

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FIG. 1. Polarographic assay of respiration and oxidative phosphorylation in rat mitochondria.

FIG. 2. Respiratory stimulation caused by o-salicylate and DNP in rat mitochondria.

rotating cuvette in which the reaction was carried out. Initial oxygen concentration (240 μM) is indicated by level of recorded trace on the extreme left. An aliquot of mitochondrial suspension, sarcosomes, was then added, and the next portion of the trace shows slow rate of endogenous substrate respiration. The system was then saturated with substrate, KG, and after several seconds rate of oxygen consumption calculated from slope of curve was 0.56 μM/sec. Addition of phosphate acceptor, ADP, caused an approximately 5-fold increased rate of respiration, which proceeded linearly for about 10 seconds. After this, the original rate of KG respiration was approximated and it was presumed that the added ADP had been converted to ATP at the point where respiration declined. It can be calculated that 41 μM O_2 (or 82 micro-atoms liter) were consumed during conversion of 250 μM ADP to ATP, giving an ADP/O ratio of 250/82 or 3.1. A second addition of ADP produced similar results, although a somewhat lower ADP/O ratio was obtained (2.8) due to small amount of ATPase activity. The presence of ATPase activity is suggested by the more rapid rate of KG respiration (1.05 μM/sec.) following second ADP addition(19). Adding multiple small doses of ADP in a single experiment permits many determinations of the efficiency as well as rate of phosphorylation. Uncoupling of oxidative phosphorylation also causes increased rate of respiration(14). The increased slope following addition of DNP in Fig. 1 indicates an approximate 5-fold increase over the initial rate of KG respiration. Exhaustion of oxygen in the reaction mixture is revealed by a horizontal trace. With this method it was possible to assess the effect of a suspected uncoupling agent on respiration several seconds after its addition to a mitochondrial suspension.

Effect of salicylates and related compounds on respiration. The procedure for testing these compounds is shown in Fig. 2. At start of experiment KG was added to the mitochondrial suspension in the reaction mixture. Respiration on KG proceeded several seconds until it was possible to calculate this initial rate of oxygen consumption (A). Salicylate was then added, and it can be seen that 2.50 mM salicylate increased the rate of linear respiration to 1.37 μM/sec. (B). Respiratory stimulation caused by salicylate, calculated from the B/A ratio, was a 2.4-fold increase. Addition of DNP caused further increase in respiration to 1.77 μM/sec. (C). Total respiratory stimulation caused by salicylate plus DNP (C/A) was 3.8-fold. Further stimulation caused by DNP in Fig. 2 indicates that the amount of salicylate added (2.50 mM) was insufficient to fully uncouple oxidative phosphorylation. Thus, by varying the concentration of a suspected uncoupler a sensitive assay is available for comparing its effect with a known uncoupling agent. With this procedure a variety of salicylates and related compounds were examined for their effect on KG respiration (Table I) in a phosphate-acceptor deficient system. The concentrations were less than that required for maximal stimulation (except for DNP). ADP was tested as control for full respiratory stimulation in the presence of phosphate-acceptor. Of monohydroxy benzoates tested, only the ortho compound stimulated respiration as shown by the B/A ratios; meta and para compounds were inactive (Table I). Acetylsalicylates produced approximately the same stimulation as sodium salicylate. Results with

TABLE I. Effect of Salicylates on Respiration of Alpha-ketoglutarate in Rat Heart Mitochondria.

(A) KG respiration, μM O_2/sec.	Second addition	Conc., mM	(B) Subsequent respiration, μM O_2/sec.	Respiratory stimulation (B/A)
.39	None		.39	1.0
.32	2-hydroxybenzoate (salicylate)	2.50	.83	2.6
.32	3- ” ”	”	.28	.9
.48	4- ” ”	”	.58	1.2
.45	2,4-dihydroxybenzoate (resorcylicate)	”	.45	1.0
.47	2,5-dihydroxybenzoate (gentisicate)	”	.47	”
.53	2,6-dihydroxybenzoate	”	.53	”
.28	3,5- ”	”	.28	”
.56	2-mercaptobenzoate (thiosalicylate)	.30	4.11	7.4
.42	acetylsalicylate	1.75	.88	2.1
.39	2-hydroxybenzaldehyde (salicylaldehyde)	1.25	.56	1.4
.50	2-hydroxybenzamide (salicylamide)	2.50	.56	1.1
.35	methylsalicylate (wintergreen oil)	5.00	.26	.7
.45	salicyl ureate	2.50	.56	1.2
.34	5-sulfosalicylate	”	.32	.9
.34	benzene thiol	.10	.34	1.0
.71	phenol	.50	.87	1.2
.35	L-thyroxine	”	.88	2.5
.40	triiodothyronine	”	1.04	2.6
.70	2,4-dinitrophenol	.10	7.72	11.0
.60	adenosine diphosphate	.50	7.00	14.0
.65	benzoate	.50	.74	1.1

dihydroxy benzoates were uniformly negative; these compounds neither stimulated nor inhibited respiration at concentrations employed. Similarly salicylate derivatives such as sulfosalicylate, salicyl ureate, salicyl amide, and salicyl aldehyde showed very small or no stimulation. Methyl salicylate was inhibitory when tested at 5 mM concentration; lower concentrations were initially without effect, but as time of incubation increased, inhibition of respiration was observed. The response to thiosalicylate was considerably greater than that to salicylate. Table I also shows that some parent compounds of the salicylate and thiosalicylate, such as phenol, benzoate, and benzenethiol had no effect on respiration at concentrations used. Thyroxine and triiodothyronine readily accelerated respiration.

The effect of salicylate and thiosalicylate was investigated in more detail (Table II). With the thiol compound, a measurable stimulation of respiration can be observed with as little as a 5 μM concentration. In one case, the concentration required to give approximately the same respiratory stimulation (3.70 and 3.85 respectively) was 2.50 mM for the former and 0.10 mM for the latter. In parallel experiments it was shown that 0.25 mM thiosalicylate decreased ADP/O ratio of controls from 3.59 to 2.63, a 27% inhibition of phosphorylation. In these experiments thiosalicylate was added to ADP and the experiment performed as in Fig. 1 for ADP only. This result is in accord with the assumption that extent of respiratory stimulation by an uncoupling agent is inversely related to efficiency of phosphorylation.

Discussion. Salicylate, acetylsalicylate, and thiosalicylate increased respiration in rat

TABLE II. Effect of Salicylate and Thiosalicylate on Respiration of Alpha-ketoglutarate by Rat Heart Mitochondria.

Conc., mM	Respiratory stimulation (B/A) Salicylate	Respiratory stimulation (B/A) Thiosalicylate
.001	1	1
.005	1	1.80
.01	1	2.10
.04	1	2.40
.10		3.85
.30		7.35
.50	1.65	11.90
1.50	2.20	
1.75	2.40	
2.50	3.70	
3.00	5.80	

heart sarcosomes whereas a variety of salicylate analogs were inactive. Hydrolysis of acetylsalicylate in alkaline solution accounts for similar activity of sodium salicylate and acetylsalicylate. Enhanced activity of thiol compound relative to phenol may be due to the more acid nature of the thiol compound. This would also explain why salicylate (o-hydroxy benzoic acid) was active and m- and p-hydroxy benzoic acids were not, since their pK's are 2.98, 4.08, and 4.58 respectively. Anthranilic acid, pK 11.3, was also inactive. On the other hand, such considerations do not adequately explain the inactivity of dihydroxy derivative resorcylic acid, which has a pK even lower than that of salicylate(20). Moreover, these conflicting results are not completely explained by studies of DeDeken(21) who showed that degree of uncoupling in yeast was correlated with several factors: mobility of the proton, compounds with low pK values being most active; a low extracellular pH, favoring undissociated form of the compound and better penetration; and high liposolubility serving to enhance penetration. Though the lesser liposolubility of the dihydroxy benzoic acids might be invoked to explain the inactivity of resorcylic acid, this seems unlikely, and additional factors such as steric hindrance, ability to activate ATPase activity, and many others may play a role.

Salicylate, like triiodothyronine, is capable of uncoupling oxidative phosphorylation in heart mitochondria, but unlike triiodothyronine, does not increase heart rate in the intact animal, suggests that thyroid hormone augments heart rate by some means other than increased tissue oxygen consumption. This is consistent with the evidence that thyroid hypermetabolism but not salicylate-induced hypermetabolism increases responsiveness of the cardiovascular system to endogenous epinephrine and norepinephrine(10).

As pointed out by Brody(6) the fact that gentisic and resorcylic acid does not uncouple oxidative phosphorylation but are apparently anti-rheumatic(22-23) is inconsistent with the suggestion that uncoupling is an essential property of an anti-inflammatory agent. Not only are there clinical studies suggesting that resorcylic acid is anti-rheumatic, but also there are animal experiments indicating that it is anti-inflammatory(22). Furthermore, dinitrophenol, a potent uncoupling agent, is not anti-inflammatory(16). Hence, the bulk of the available evidence does not support the contention that uncoupling is an essential property of an anti-inflammatory or anti-rheumatic drug.

Summary. 1) The polarographic method for measuring changes in O_2 concentration has been employed for assessing ability of salicylates and related compounds to uncouple phosphorylation. The extent of stimulation of respiration caused by addition of these compounds to rat heart mitochondria oxidizing alpha-ketoglutarate in a phosphate-acceptor deficient system was used as a criterion of uncoupling action. 2) Thiosalicylate was many times more active than salicylate and other salicylate-derivatives tested. Some chemical and physical properties possibly responsible for the uncoupling action of salicylate and certain of its congeners were considered. The *in vitro* action of salicylates was discussed in relation to their anti-rheumatic activity and cardiovascular effects.

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Effect of Prolonged Exercise on Atherogenesis in the Rabbit.* (24586)

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Morris(1) demonstrated that men who do physically active work have a lower incidence of coronary heart disease than men in sedentary jobs. The physiological basis for this finding has not been elucidated. Brown(2) found that 20 minutes of exercise/day did not inhibit atherosclerotic plaque formation in rabbits fed a high cholesterol diet, while Kobernick(3) noted that 5 minutes of exercise/day did inhibit cholesterol deposition as measured chemically in the aortae of rabbits fed cholesterol. Using young cockerels fed a high cholesterol diet, Orma(4) showed that mild exercise, possibly through its effect on the thyroid gland, inhibited atherogenesis and lowered serum cholesterol levels. The present study is designed to evaluate the effect of more prolonged exercise on cholesterol concentration in serum, aorta, muscle, liver and skin and on aortic plaque formation in rabbits fed a high cholesterol diet.

Method. Male American-Dutch rabbits 6 months old were fed a diet consisting of commercial rabbit pellets to which 0.3% of cholesterol by weight was added by dissolving in ether, mixing with the pellets, then allowing ether to evaporate. One-half of the animals were exercised daily by placing them in a revolving drum 3 feet diameter and 6 feet long. The drum was rotated by motor at a speed just sufficient to keep the animals moving with an automatic timing device which allowed alternating periods of rest and exercise every 15 minutes. All rabbits were fed high cholesterol diet and water *ad lib.* The exercise group received nothing by mouth during the 8 hours in the machine. After a few days of familiarization, the exercise group tolerated the forced activity well. Blood was drawn by cardiac puncture for cholesterol determination after 1 month, and at 2 months when experiment was terminated. The aortas were examined grossly and transverse sections through the entire circumference were taken from the proximal ascending aorta for tissue cholesterol determination. Tissue from liver, thigh muscle, and abdominal skin was similarly evaluated. Cholesterol levels of serum and tissue were determined by the method of Herrmann(5). The aortas were removed in continuity from aortic valve to the bifurcation and grossly graded for plaque formation.

Results. The results (Table I) revealed little difference between the 2 groups. Statistical analysis demonstrated a "p" value of less than 5% in only one comparative measurement—average serum cholesterol level at end of experiment. Average serum cholesterol of rabbits fed a plain diet was 59 mg %. The values of 903 mg % and 501 mg % for the exercise and non-exercise groups, respectively, on a high cholesterol diet were markedly elevated. The difference between means was significant (p = .0001).

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† Trainee, Nat. Cancer Inst.

TABLE I. Results of Exercise and Inactivity upon Rabbits Fed a High Cholesterol Diet.

	Exercise group	Non-exercise group
No. of animals	11	11
Mean wt at end of experiment, kg	1.85 ± .27	1.71 ± .24
" serum cholesterol, 9/16/57, mg %	684 ±298	465 ±228
11/20 "	903 ±278	501 ±212
" aortic cholesterol, mg/g	6.6 ± 3	4.5 ± 2
" muscle " "	.7 ± .1	.7 ± .1
" liver " "	19.8 ± 9.8	31.1 ± 14.1
" skin " "	3.9 ± 2	2.7 ± 1
" grade plaque formation	II	II

Average values of aortic cholesterol of 6.6 mg and 4.5 mg cholesterol/g of tissue for 2 groups did not differ significantly and correlated with the finding of Grade II plaque formation regardless of whether exercise was or was not given. The muscle cholesterol level was low at 0.7 mg/g of tissue and identical in the 2 groups. The skin cholesterol levels did not differ significantly. The liver cholesterol was much higher than the other tissue cholesterol levels and tended to be more elevated in the non-exercise group.

Discussion. It is evident that exercise did not inhibit deposition of cholesterol in the rabbit aorta. The only statistically significant difference between the 2 groups was the higher serum cholesterol level in the exercise group at the end of experiment. This may have resulted from increased food intake on the *ad lib.* diet. Mean weight of exercised animals was slightly higher than sedentary animals. These results differ markedly from those of Kobernick *et al.*(3). Many factors could account for the difference, such as New Zealand white rabbits of mixed sex being used by Kobernick while male American-Dutch rabbits were used in the present experiment. Also, the exercise period differed, with Kobernick using 5 min. twice a day instead of 15 min. alternating exercise and rest periods for 8 hrs. Probably the most important factor was the limited food intake of Kobernick's exercise and sedentary groups, as evidenced by all animals eating all food offered, the same quantity by weight for both groups. His exercised animals were leaner, had less body fat, and were lighter than the sedentary ones. The finding of less atherosclerosis in the exercise group can be accounted for on the basis of not providing enough calories beyond those for physical exertion.

It may be that in rabbits fed an atherogenic diet of limited quantity atherosclerosis can be inhibited by exercise. But the ingestion of *ad lib.* quantities of the same diet overwhelms the effect of exercise.

It is possible that stress of forced exercise influences cholesterol metabolism by release of cortisone (which is known to elevate blood cholesterol levels), but differences in amount of steroid release following electric shock stimulation as used by Kobernick *vs.* mechanical rotation in a drum are probably not significant.

The difficulty found by Kobernick in inducing rabbits to run in a rotating drum was not encountered, perhaps because of use of a different strain of rabbits. The protection of exercise against coronary heart disease, so clearly shown by Morris, may result from something other than inhibition of atherogenesis. Zoll, Wessler, and Schlesinger(6) have shown that relative cardiac hypoxia is an underlying factor in the etiology of human intercoronary anastomoses. There is evidence(7) that intercoronary anastomoses protect the heart against coronary occlusion. The effect of exercise may be the creation of intercoronary anastomoses by recurring periods of relative cardiac hypoxia.

There is a general correlation(2) between quantity of atheromatous deposits in the aorta and in the coronary arteries, and, in the rabbit at least, atherogenesis is clearly related to cholesterol intake. Further study might be directed toward the effect of exercise on coronary vasculature, with emphasis on intercoronary anastomoses.

Summary. Rabbits fed *ad lib.* on a high cholesterol diet were exercised 8 hours daily for 2 months. This regimen did not inhibit atherogenesis as measured by serum and aortic tissue cholesterol levels.

Acknowledgement is made of the valuable technical assistance of Miss Elizabeth Swyryd.

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Hormonal Effects on C-14 Acetate Metabolism in the Human.[†] (24587)

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An important and intriguing segment of intermediary metabolism is concerned with reactions, both synthetic and degradative, involving the common 2 carbon fragment of metabolism, "acetate." Many of these reactions can be studied in a general way in relatively undisturbed human subjects with the aid of carbon-14 labeled compounds. We studied the effect of glucocorticoid administration and diabetes on certain of the fates of 1 and 2-C^{14} labeled acetate in the human. Though some results are preliminary in nature, we believe them of sufficient interest, to report at this time.

Procedure. Well controlled diabetics served as subjects to determine the effect of 3 types of diabetes on synthesis of various lipid fractions from 1- and 2-C^{14} acetate. In an attempt to prevent a compensatory secretion of insulin which might obscure the glucocorticoid effect, the same diabetics were also used as subjects for studying the effect of glucocorticoids on utilization of C^{14} acetate. These subjects were hospitalized and were on a constant intake of carbohydrate, protein and fat. Their weights were stable for several weeks prior to each experiment. Their usual dose of long acting insulin was given 24 hours prior to the experiment and (except for the stable adult diabetic (D.M.) following steroid administration), each subject had a normal fasting blood sugar and was non-ketotic at the start of each experimental procedure. Thirty mg of prednisone (delta-1-cortisone) was given orally 9 and 3 hours prior to several experiments, to study glucocorticoid effect on utilization of acetate. No food was allowed on day of experiment until 8 hours from start of procedure, at which time the usual evening meal was given. Interval between experiments in the same subject was 1-3 months. Breath samples for respiratory $C^{14}O_2$ excretion, blood samples for pyruvic and alpha ketoglutaric acids, lipids, ketones, and glucose were collected at various intervals after administration of 40-100 microcuries of 1 or 2-C^{14} acetate intravenously. Respiratory CO_2 was trapped by the method of Baker, Shreeve *et al.*(1). Specific activity of respiratory CO_2 was determined by the method of Van Slyke *et al.*(2). Specific activity of pyruvic and alpha ketoglutaric acids was determined as follows: Whole blood keto acids were determined by paper chromatographic method of Seligson and Shapiro(3). Pyruvic and alpha keto glutaric acids were determined quantitatively. A portion of the eluate from the paper strip was transferred to Van Slyke combustion tube and dried. Radioactivity was then determined by wet carbon combustion and determination of radioactivity of the evolved CO_2 by the method of Van Slyke *et al.*(2). Lipid samples were chromatographed on silicic acid columns by a modification of the method of Fillerup and Mead(4). Triglycerides were determined colorimetrically on an eluate from the column, by the method of Stern and Shapiro(5). Cholesterol was determined quantitatively on an eluate from the column by the method of Carr and Drekter(6). After saponification, extraction, and washing, another portion of the eluate from each major lipid fraction studied was mixed with 15 ml of 2-phenyl-5 (4 biphenyl) 1,3,4,-oxiadiazole 0.6% (PBD) in toluene and radioactivity was determined in a Packard Tri-Carb liquid Scintillation counter. All results have been corrected to the same amount of administered radioactivity/kg body weight.

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Results. In each of 4 studies, administration of prednisone has been associated with a 10-15% decrease in 24 hour cumulative excretion of radioactivity in respiratory CO_2 as compared to the control experiment in the same subject(Fig. 1). The most striking changes are seen in early breath samples which show as much as 20-30% decrease in specific activity as compared to the control experiment. Administration of prednisone has been associated consistently in these experiments with decrease in peak specific activity of CO_2, a late peak in activity and a decrease in cumulative excretion of C-14 in respiratory CO_2. Logarithmic decline of specific activity is essentially parallel in the 2 series of experiments. These findings suggest that the effect of adrenal steroids in decreasing respiratory $C^{14}O_2$ following administration of C^{14} acetate is not due to enlargement of body CO_2 pool, since the straight line logarithmic fall off in specific activity is thought to be a measure of this pool. Rather, these findings suggest that glucocorticoids may exert some effect on initial disposition of acetate which decreases its early oxidation to CO_2.

We also compared respiratory $C^{14}O_2$ following administration of carboxyl and methyl labeled acetate to the same subject. Previous *in vitro* experiments with plant seeds and animal tissues and *in vivo* experiments with lactating cows have shown that carboxyl carbon of acetate is more rapidly oxidized to CO_2 than is methyl carbon and total CO_2 production from carboxyl carbon is much greater. This difference can be readily demonstrated in the human (Fig. 2). As pointed out by Weinman, Strisower, and Chaikoff(7), this phenomenon can be partially explained on the basis of known reactions of the Krebs Cycle. The carboxyl carbon of acetate is oxidized in one turn of the cycle while methyl carbon is not. However, since oxidation of acetate occurs almost explosively and radioactivity is rapidly randomized in the Krebs cycle acids, oxidation of methyl carbon should speedily approach that of the carboxyl carbon in an isolated Krebs cycle. Since production of respiratory $C^{14}O_2$ from methyl labeled acetate does not approach that from carboxyl labeled acetate during the 24 hour period of study, it can probably be assumed that Krebs cycle acids readily and rapidly enter other compounds which are less rapidly oxidized. Perhaps due to its longer stay in the Krebs cycle acids, the methyl carbon of acetate enters these compounds more extensively than does the carboxyl carbon. Regulation of amounts of Krebs cycle substrates going to such pools may be an important means of regulating energy production in the cell.

As perhaps another measure of cellular oxidative processes, we determined the specific activity of whole blood pyruvic and alpha ketoglutaric acids following administration of C^{14} acetate. In almost all of approximately 60 samples analyzed specific activity of alpha ketoglutarate has been higher than that of pyruvate, undoubtedly reflecting the dilution of radioactive pyruvate, derived probably from previously intra-mitochondrial oxaloacetate, with non-radioactive pyruvate resulting from glycolysis.

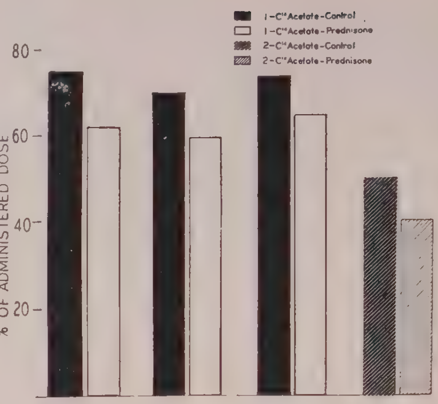
Incorporation of 2-C^{14} acetate into free cholesterol, ester cholesterol and triglyceride fatty acid was studied in a young unstable and in an adult stable diabetic before and after administration of prednisone. Though specific activity of the earliest triglyceride samples was increased in both subjects following steroid administration, there was an associated decrease in absolute levels of plasma triglycerides, so that total incorporation of radioactivity into plasma triglycerides was actually diminished by 25-50%. These preliminary findings suggest that adrenal 17-hydroxycorticoids in high dosage may decrease mobilization of fat from the depots.

No striking differences in specific activity of early samples of plasma free cholesterol were noted after acute administration of prednisone. There is a suggestion of a later decrease in specific activity following prednisone. These studies do not show the striking increase in cholesterol specific activity noted by Gould *et al.*(8), following prolonged administration of cortisone to chronically ill patients. It seems likely, therefore, that an increased synthesis of cholesterol under the influence of adrenal steroids depends on secondary metabolic changes and is not a primary effect of administration of adrenal 17 hydroxycorticoids.

A comparison was also made between specific activity of these lipid fractions following administration of 1- and 2-C^{14} acetate to the same subject in a comparable state of diabetic control. Since much more C^{14} was present in expired CO_2 following administration of carboxyl labeled than methyl labeled acetate, we expected to find greater radioactivity in lipids synthesized from methyl labeled than from the carboxyl labeled compound. This was not the case. Actually, the specific activity of triglycerides and cholesterol was approximately twice as great following administration of carboxyl labeled acetate, despite the fact that much less radioactive acetate should have been available for synthetic purposes as indicated by respiratory $C^{14}O_2$. Feller has found in *in vitro* studies that specific activity of the fatty acids of rat adipose tissue is approximately 2 times as great following administration of carboxyl labeled acetate as it is following administration of the methyl labeled compound(9).

TABLE I. Peak Specific Activity of Plasma Lipids following I. V. 2-C^{14} Acetate.

	DPM/mg	
	Triglyceride fatty acids	Free cholesterol
Lipoatrophic diabetic	271	440
Young unstable "	235	242
Middle aged control	110	111
Stable adult diabetic	44	78



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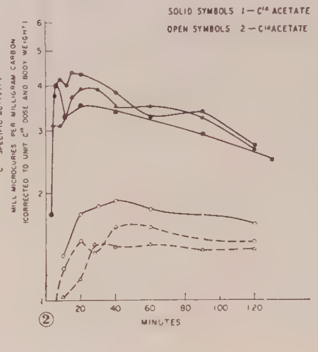

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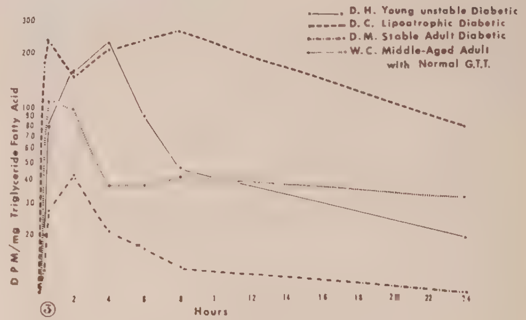

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FIG. 1. Effect of prednisone on 24-hr cumulative excretion of $C^{14}O_2$ from C^{14} acetate.

FIG. 2. Comparison of specific activity of respiratory CO_2 following administration of 1- and 2-C^{14} acetate to same subject.

FIG. 3. Specific activity of plasma triglyceride fatty acids following administration of I.V. 2-C^{14} acetate.

We studied lipid synthesis from 2-C^{14} acetate in each of Lawrence's 3 types of diabetics (10) (Table I). Because 2-C^{14} acetate was administered intravenously in these studies, whereas previous studies of triglyceride synthesis in non-diabetics by Lipsky *et al.*(11) have been done following oral administration of 1-C^{14} acetate, only a very general comparison of these studies with those of Lipsky can be made.

In a well controlled young unstable diabetic (D. H., age 27) triglyceride synthesis is quite rapid with a peak approximately $\frac{1}{6}$-$\frac{1}{2}$ that found by Lipsky. The general shape of the curve of rise and decline in specific activity is similar to that found by Lipsky (Fig. 3). In a 14-year-old girl with classical lipoatrophic diabetes† (12) peak specific activity is reached more rapidly, but is about the same level as found in the young unstable diabetic. However, a high level of activity in triglycerides persists longer than normal. Thus, the striking loss of adipose tissue in this patient does not seem to be due to an increased utilization of fat, since a more rapid disappearance of radioactivity than normal should have been seen if this were the case. The persistence of high specific activity in plasma triglycerides may be due both to less dilution with unlabeled fat from the adipose tissue and to less deposition of labeled fat in adipose tissue. It seems likely that individuals with this condition are unable to store and perhaps to synthesize fat in the adipose tissue. The findings in this patient seem quite compatible with Lawrence's suggestion that the metabolic defect in this syndrome is primarily in the adipose tissue and that "diabetes" in these patients may be a symptom of inability of the adipose tissue to use carbohydrate for synthesizing fat. Oxidation of 2-C^{14} acetate was not grossly different in pattern from that in other diabetics.

The stable adult diabetic shows a surprisingly low level of radioactivity in plasma triglyceride fatty acids. Peak specific activity is much lower than in either of the 2 young diabetics and is very much lower than has been noted in individuals without diabetes(11).

Because it seemed possible that the striking decrease in specific activity of plasma triglycerides found in the stable adult diabetic might be an effect of age, rather than of diabetes, a similar study was done in an adult of same age and body build, but with normal glucose tolerance. This subject showed much less active triglyceride synthesis than did the young diabetics. However, specific activity of plasma triglycerides was approximately $2\frac{1}{2}$ times greater than in the stable adult diabetic. It will be of extreme importance in understanding diabetes to determine whether a decrease in ability to synthesize fatty acids from acetate is a consistent defect in the stable adult diabetic with normal fasting blood sugar as compared to younger individuals and to subjects in the same age group. It is interesting that Shreeve *et al.*(13) have found no decrease in the portion of C^{14} glucose oxidized to CO_2 in the stable adult diabetic. It is tempting to speculate, as Lawrence already has, that the defect in glucose utilization in the obese adult diabetic may be one in conversion of carbohydrate to fat. However, much work will be required before this postulate can be confirmed or denied.

The synthesis of plasma free cholesterol was also studied in these same patients (Table I). The young unstable diabetic under good control attains a peak specific activity quite comparable to the average found by Gould *et al.* with 1-C^{14} acetate(8). Peak specific activity in the young girl with lipoatrophic diabetes is approximately twice as great. The middle aged adult with normal glucose tolerance attains a peak specific activity which is just at the lower limits of Gould's range. The stable adult diabetic shows an even lower peak specific activity, being greater than 2 S.D. from Gould's average value. However, because of limitation of dose of C^{14} in normal individuals of all ages, and particularly in young individuals, it may be many years before normal ranges of lipid synthesis from 1- and 2-C^{14} acetate in various age groups can be established with certainty.

Incorporation of radioactivity into ester cholesterol lags behind that of free cholesterol since it is formed from it. The differences in radioactivity in ester cholesterol between these patients merely reflect and confirm the previously noted differences in plasma free cholesterol.

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† Studied with help of Robert W. Schwartz, Boston Children's Hosp.

Summary. These data suggest the following tentative conclusions: 1. Adrenal 17-hydroxycorticoids appear to exert an effect on utilization of acetate and to decrease oxidation of this 2 carbon fragment to CO_2. 2. In the human as in tissues of lower animals and in plant seeds, carboxyl carbon of acetate is more readily oxidized than is methyl carbon. The finding of a decrease in lipid synthesis form methyl labeled acetate in association with a decrease in oxidation of this compound as compared to carboxyl labeled acetate suggests that the carboxyl carbon may also be used preferentially for lipid synthesis in the human. 3. The syndrome of lipoatrophic diabetes appears to be associated with a decreased ability to store and perhaps to synthesize lipids in the adipose tissue rather than an increase in utilization of lipids of adipose tissue. 4. The stable adult diabetic may have a defect in conversion of 2 carbon fragments to fatty acids, even when the fasting blood sugar is normal.

Mr. Tanous Faris rendered able technical assistance during summer fellowship. The authors gratefully acknowledge helpful suggestions of Dr. Donald D. Van Slyke.

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Effect of Dietary Sucrose and Glucose on Plasma Cholesterol in Chicks and Rabbits.* (24588)

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The influence of certain components of diet on level of cholesterol in blood has been the subject of much investigation and speculation. That type of protein used may alter cholesterol level was indicated as early as 1940(1), and the association of different types of fat with various serum cholesterol values has also been described(2). In many such studies the effect of carbohydrate has not been considered. However, it has recently been reported (3) that cholesterol-fed rats developed significantly higher serum cholesterol levels when sucrose was substituted for starch in the diet. A similar but less pronounced effect was noticed when sucrose replaced glucose. The purpose of this study was to investigate the difference between glucose and sucrose in regulation of plasma cholesterol in chicks and rabbits.

Methods. Day-old single-comb White Leghorn male chicks‡ were given water and purified basal diets (Table I) *ad lib*. with appropriate supplementation for 28 days. At end of this period blood was removed by heart

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‡ Obtained from Kerr Chickeries, Frenchtown, N. J.

TABLE I. Purified Basal Diets for Chicks.

(a) Constant ingredients

	g		mg
Corn oil	3	Thiamine	1
Ca gluconate	5	Riboflavin	1
Salt mixture	2*	Niacinamide	5
Bone ash	2	Pyridoxine	1
Choline chloride	.2	Ca pantothenate	5
Inositol	.1	Menadione sodium bisulfite	.5
		Folic acid	.5
		Tocopherol acetate	.5

	Units		µg
Vit. A	1000	Biotin	20
D_3	200	Vit. B_{12}	5

(b) Variable ingredients

	Diet 1 (g)	Diet 2 (g)	Diet 3 (g)
Sucrose	0	60	0
Glucose†	64	0	60
Casein	0	20	20
Gelatin	0	8	8
Soybean protein‡	25	0	0
L-cystine	0	.4	.4
DL-methionine	.7	0	0
Cholesterol	0 or 3	0 or 3	0 or 3

* Final concentration of salts in the diet: NaCl 0.6, K_2HPO_4 0.6, KH_2PO_4 0.45, $MgSO_4$ 0.25, $MnSO_4 \cdot H_2O$ 0.04, $FeC_6H_5O_7 \cdot 3H_2O$ 0.05, $CuSO_4 \cdot 5H_2O$ 0.002%. KI 6, $Zn(C_2H_3O_2)_2$ 14, $Al_2(SO_4)_3 \cdot 18H_2O$ 16, $Co(C_2H_3O_2)_2 \cdot 4H_2O$ 4, $NiCl_2 \cdot 6H_2O$ 2, KBr 8, $Na_2MoO_4 \cdot 2H_2O$ 0.5 parts/million.

† Cerelose.

‡ Assay protein C-1, Archer-Daniels-Midland Co., Cincinnati, O.

puncture, heparinized and the plasma analyzed for cholesterol(4). Chicks were treated in groups of 8 to 10. Male Dutch Belted rabbits,§ weighing 1.5 to 2.0 kg, were maintained for 60 days in individual cages. They were fed a purified diet containing soybean protein 25%, methionine 0.3%, cellophane 'spangles' 10%, corn oil 5%, cholesterol 1%, and choline chloride 0.15% with complete vitamin and salt mixture(5). One group of 15 was given 50% glucose while the other had 50% sucrose in the diet. Blood was removed by heart puncture for cholesterol analysis(4).

Results. Plasma cholesterol of 7 groups of chicks which were fed Diet 1 with cholesterol 3% reached average level of 296 mg % after 28 days (Table II). Seven other groups receiving Diet 2 with cholesterol 3% developed average plasma cholesterol value of 601 mg %, which was significantly ($p<0.01$) higher than the average value on glucose-soybean protein diet. When these diets were fed without added cholesterol, average cholesterol values were 170 mg % and 177 mg % respectively.

To determine whether a similar difference in plasma cholesterol concentration would result

TABLE II. Effect of 2 Purified Diets on Plasma Cholesterol Levels of 28-Day-Old White Leghorn Cockerels.

Added dietary cholesterol (%)	Plasma cholesterol levels* (mg % ± S.E.) of chicks fed	
	Diet 1†	Diet 2†
0	190 ± 19	169 ± 28
	172 ± 22	184 ± 80
	136 ± 20	219 ± 42
	214 ± 38	192 ± 42
	162 ± 20	147 ± 32
	147 ± 30	155 ± 56
Mean	170 ± 27	178 ± 24
3	346 ±141	636 ±310
	242 ± 59	428 ±194
	241 ± 54	721 ±369
	338 ±118	635 ±165
	353 ±117	425 ±116
	326 ±114	645 ±120
	228 ± 72	714 ±185
Mean	296 ± 54	601 ±113

* Each cholesterol value represents a single experiment and is avg plasma cholesterol level resulting from individual determinations on 8 to 10 chicks.

† See Table I.

TABLE III. Comparative Effects of Glucose and Sucrose on Plasma Cholesterol Values of 28-Day-Old White Leghorn Cockerels Fed Purified Diets.

Added dietary cholesterol (%)	Plasma cholesterol levels* (mg % ± S.E.) of chicks fed	
	Diet 3†	Diet 2†
0	110 ± 31	155 ± 56
	242 ± 23	179 ± 19
	183 ± 51	192 ± 42
	175 ± 95	147 ± 32
	118 ± 4	
Mean	166 ± 47	168 ± 20
3	360 ± 73	645 ±120
	486 ±118	752 ±134
	452 ±189	635 ±165
	380 ±153	425 ±116
	470 ±178	714 ±185
Mean	430 ± 46	634 ±114

* Each cholesterol value represents a single experiment and is avg plasma cholesterol level resulting from individual determinations on 8 to 10 White Leghorn cockerels after 28 days on experimental diets.

† See Table I.

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§ From Research Animal Center, Nanuet, N. Y.

TABLE IV. Effect of Glucose *vs* Sucrose on Plasma Cholesterol Values of 28-Day-Old White Leghorn Cockerels Fed a Crude Diet.

Added dietary cholesterol (%)	Plasma cholesterol levels* (mg % ± S.E.) of chicks fed a crude diet† containing	
	Glucose 50%	Sucrose 50%
0	113 ± 1.4	113 ± 7.9
3	359 ±131.3	627 ±197.0

* Each plasma cholesterol value is avg of individual determinations on 10 White Leghorn cockerels after 28 days on experimental diets.

† Composition of diet: Fish meal 25, fish solubles 5, alfalfa meal 2, casein 5, distillers solubles 5, choline chloride .05, corn oil 3, carbohydrate 50, NaCl 0.25, Ca 0.33, Mn 0.002, I 0.00045%; vitamins were same as employed in purified basal diet, Table I.

when only the carbohydrate component was varied, the Diets 2 and 3 were compared. After 28 days on these diets with 3% added cholesterol, 5 groups on glucose supplemented diet had cholesterol values averaging 430 mg % while 5 on sucrose had 634 mg %. This was a significant difference ($p<0.01$). When no cholesterol was added, values were 166 mg % and 168 mg % respectively.

The effect of glucose or sucrose in another diet containing varied and cruder protein sources is shown in Table IV. As in the previous experiment when glucose was the carbohydrate, in the presence of 3% cholesterol plasma cholesterol values averaged 359 mg % after 28 days, whereas with sucrose the value was 627 mg % ($p<0.02$). When no cholesterol was added, plasma cholesterol levels were not significantly different.

In experiments with rabbits, 2 groups of 15 male Dutch Belted rabbits were fed a diet containing soy protein; one group received glucose 50% and the other sucrose 50% as sole carbohydrate. After 60 days the rabbits on glucose diet had an average plasma cholesterol concentration of 1015 mg % while those on sucrose had 1539 mg % ($p = 0.02$).

Discussion. Higher plasma cholesterol levels were obtained in chicks and rabbits when sucrose was substituted for glucose as the sole source of carbohydrate in various purified diets. A preliminary trial with fructose as the only dietary carbohydrate suggested that it produced extremely high cholesterol levels in chicks but this experiment was unsatisfactory because of pronounced growth depression.

One may speculate that sucrose favors absorption of exogenous cholesterol. This reasoning is suggested by the observation that there was no difference in plasma cholesterol when either glucose or sucrose was fed without added cholesterol.

Summary. Chicks were fed purified diets containing 3% cholesterol and either casein, gelatin and sucrose or soybean protein and glucose together with vitamins and minerals. The chicks receiving casein, gelatin and sucrose had higher plasma cholesterol values than did those whose diet contained soy protein and glucose. When both diets contained casein and gelatin, those chicks on the diet containing sucrose as sole carbohydrate source had higher plasma cholesterol values than did those on a diet containing glucose; when these same diets were fed without the addition of 3% cholesterol, no significant difference in plasma cholesterol concentrations was found. Serum cholesterol values of rabbits fed a cholesterol diet containing sucrose were higher than those from a group on a similar diet containing glucose.

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Age Changes in Swelling Properties of Human Myocardium.* (24589)

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In aging humans there is a progressive decrease in cardiac output, and cardiac failure is precipitated with increasing ease. These and other age-related changes are usually considered a result of arteriosclerotic heart disease, although there is often a poor correlation between degree of coronary arteriosclerosis and cardiac function(2). This lack of correlation has led us to the hypothesis that there might be some underlying age-related change in cardiac muscle, probably at the molecular level. Studies on osmotic swelling properties of human collagen have shown that collagen passes through several stages of increasing rigidity with advancing age, and evidence was presented suggesting such changes are due to thermal denaturation at body temperature(3, 4). Since the same factors influencing collagen might be expected to act on fibrous proteins of muscle, a study of relationship of age to swelling properties of human myocardium was undertaken.

Methods. One hundred and forty-eight hearts were obtained from autopsies performed the day of death. Only samples of left ventricle showing no macroscopic lesions were used. Epicardium and endocardium were dissected away and the remaining myocardium was stored at –60°C until used. Control experiments showed that myocardium frozen 1 to 7 days did not differ in swelling ability from that of fresh unfrozen tissue. Each sample of myocardium was cut into three 25-40 mg fragments, washed briefly in 0.9% NaCl, blotted firmly dry and weighed. The fragments were then placed in 10 ml of distilled water adjusted to required pH levels by addition of NaOH or HCl where they were maintained at room temperature for varying time. After removal, the fragments were again blotted firmly dry and weighed. Percentage change in weight during incubation was then calculated, and used as a measure of swelling. pH values below 10 were determined with glass electrode pH meter, above 10 by titration with standard acid. Because changes in swelling might be due to a restricting influence of increased connective tissue, collagen content of hearts was determined by the method of Neuman and Logan(5). Hydroxyproline determined by this method was multiplied by 7.35 to obtain amount of collagen. As changes in water content would also influence swelling ability, dry weight of myocardium was determined after drying to constant weight at 110°C.

Results. Fig. 1 shows amount of swelling over wide pH range of samples from young and old heart, incubated in the various solutions for 3 hours. Values between pH 3 and 10 are not included because these pH's were very unstable during incubation of heart fragments and swelling was negligible in this range. There are small swelling peaks at pH 0.9 and 2.4, and maximal swelling at pH 11.2. A significant age difference is apparent at pH 11.2 where the young specimen shows more swelling than the old. This pH was used for subsequent studies. At pH 11.2 maximal swelling occurred on 3 hours incubation and this time was used for the following determinations. Swelling at pH 11.2 was completely reversed when fragments were returned to neutral salt solution. Triplicate determinations were usually within ± 3% of the mean value.

Swelling of heart muscle was determined for the entire age range at pH 11.2. Results are shown in Fig. 2. There is a sharp decrease in swelling between birth and approximately 8 years of age. Extent of swelling apparently remains at constant level between 8 and approximately 35 years of age, after which there is a decrease in swelling ability to a level which appears to remain constant until old age. Beyond the fourth decade the points show considerable scatter. The dashed line of Fig. 2 represents a subjective evaluation of changes which appear to take place as a function of age.

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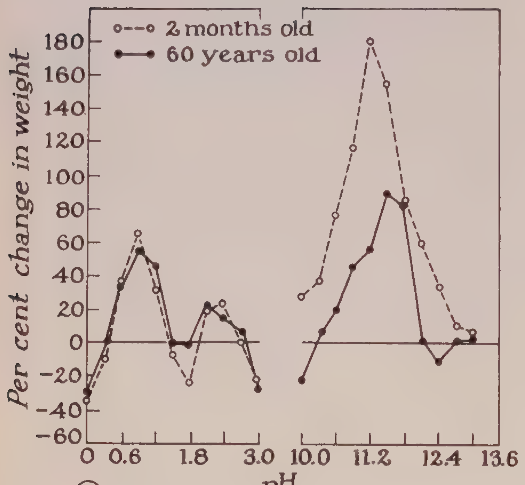
* Supported by grant from USPHS.

† USPHS Fellow in Pathology.



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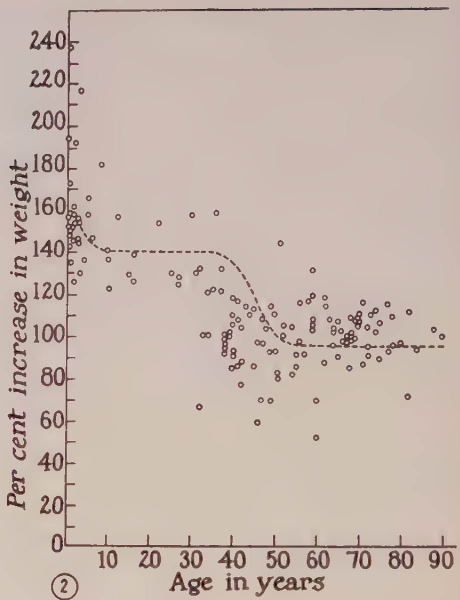

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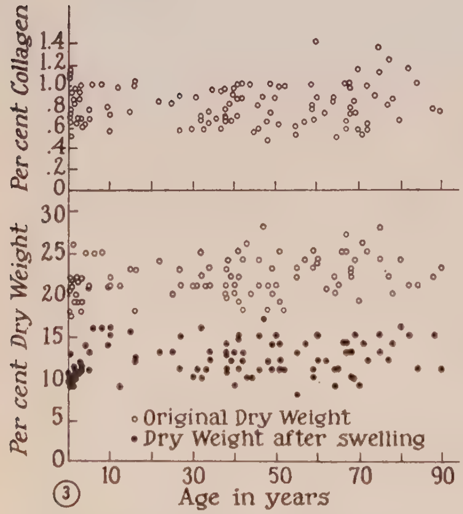

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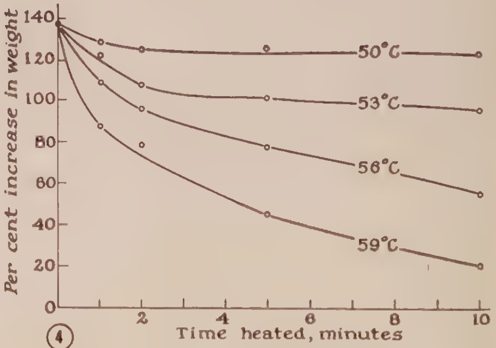

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FIG. 1. Swelling of heart fragments from old and young individuals on exposure to acid and alkaline solutions for 3 hr.

FIG. 2. Swelling of myocardium from individuals of various ages at pH 11.2.

FIG. 3. Collagen content and dry wt of myocardium as a function of age; expressed as % of original wet wt.

FIG. 4. Effect of heating myocardium on subsequent ability to swell in a pH 11.2 solution at room temperature.

Data in Fig. 3 indicate that collagen content and percentage solids do not vary with age. Percentage dry weight, based on original weight, was also determined on fragments which had swollen at pH 11.2. This was done because of the possibility that old myorcardium swelled less than young because some substance responsible for swelling was more soluble in old muscle and was extracted by the alkaline solution. These determinations are included in Fig. 3 and show that about 45% of the solids are extracted during the swelling process. The amount does not vary with age.

To find if loss in swelling with age could be caused by thermal denaturation, the effect of heat treatment on swelling was investigated. Heart fragments were incubated at elevated temperatures in mammalian Ringer's solution for various lengths of time. They were then cooled to room temperature and swelling ability at pH 11.2 was determined. Results of such an experiment with heart fragments from a 15-year-old individual are shown in Fig. 4. Heat has a marked effect in decreasing swelling ability. Control experiments showed that heat treatment did not result in extraction of solids beyond the amount extracted during swelling, and had no effect on amount of solids extracted during swelling. From data of Fig. 4 and similar data for other hearts, Arrhenius plots were constructed. These yielded activation energies of 40, 47 and 47 kcal/mol for myocardium specimens from individuals aged 15, 27 and 30 years respectively.

Discussion. Swelling properties of myocardium have apparently not been described previously. The swelling maximum at pH 2.4 is probably due to osmotic swelling of collagen in the heart, as previous studies have shown that human collagen swells maximally at this pH(3). Swelling at pH 0.9 and 11.2, in showing a decrease below pH 0.9 and above pH 11.2, is characteristic of Donnan equilibrium type of protein swelling described by Procter and Wilson(7). Collagen does not swell maximally at these pH levels and the small amount of collagen in the heart is not enough to cause swelling noted at pH 11.2. Thus, swelling at pH 0.9 and 11.2 probably results from formation of ionized myocardial protein salts.

Decrease in ability to swell at pH 11.2 in early years of life may represent part of a developmental process by which fibrous proteins become oriented and strengthened. The decrease beginning in the fourth decade, however, probably represents a degenerative process starting after maturity is attained. Loss of swelling with age implies development of some factor which increases rigidity of the protein structure. Since collagen content does not change with age, a finding in accord with the study of Oken and Boucek(6), it is possible that the change is in myocardial protein *per se*. As swelling is determined by 55% of solids which are not extracted in strongly alkaline solution, insoluble fibrous myocardial proteins may be the molecules which undergo change with age. Loss of swelling ability could be due to either a change in the type of proteins synthesized or a change in structure of relatively stable proteins. While there is no evidence that a different type of protein is or is not synthesized, there is some reason to believe that there are relatively stable molecules in the heart which might undergo changes with advancing age. Friedberg, Tarver and Greenberg, using labeled methionine, found turnover rate of myocardial proteins very low(1). Since their values applied to pooled proteins, some heart proteins must have extremely low turnover rates. Such molecules might be susceptible to the influence of various chemical and physical factors acting over many years.

A result of such influence might be an unfolding of protein molecules with formation of new cross links. Since heat treatment causes a decrease in swelling ability similar to that in aging, possibly the change in aging is due to thermal denaturation at body temperature. Using energies of activation experimentally determined at elevated temperatures and kinetics of a first order reaction, it was calculated that the loss in swelling between 35 and 55 years of age should take place in several hours at 37°C. Thus, the issue is not if body heat could cause the change, but rather what prevents the change from occurring at a more rapid rate.

A relationship between loss of swelling of myocardial proteins and decreased ability of heart to contract efficiently can be hypothesized on grounds that swelling ability is a measure of protein elasticity(7).

Summary. To detect changes with age in structure of myocardial proteins, swelling properties of human myocardium in solutions of HCl and NaOH were investigated. Fragments of myocardium were incubated in solutions of various pH values and increases in weight were used as measures of swelling ability. Maximal swelling occurred at pH 0.9, 2.4 and 11.2. Greatest swelling, probably of the Donnan equilibrium type, occurred at pH

11.2 and this value was used for subsequent studies. Swelling decreases rapidly in the first years of life, then appears to remain constant until 35 years of age after which it decreases, to apparently remain constant at a low level. Collagen content, dry weight and amount of solids extracted during swelling do not vary with age. Heating myocardium causes a decrease in swelling ability. The possible role of thermal denaturation in myocardial aging and physiological implications of swelling changes are discussed.

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Red Cell Life Span in Newborn at Sea Level and High Altitudes. (24590)

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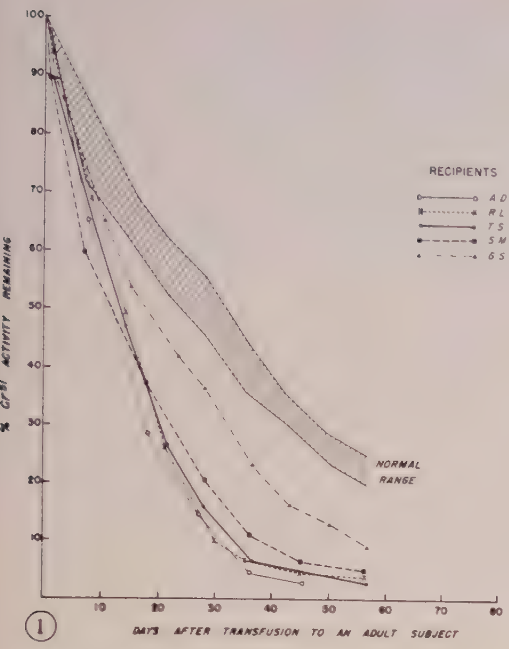
At sea level there is a marked decrease of erythrocytes in circulating blood of the newborn a few days after birth. The same process, to a lesser degree, has been described by Loret de Mola(1) in a study of newborns at high altitudes. While the decrease is chiefly due to depression in erythropoietic activity of bone marrow(2-5), a greater rate of cell destruction has been considered a contributing factor. Increased concentration of indirect bilirubin, especially during first few days after birth, has been interpreted by some investigators to favor the latter hypothesis. However, most authors believe that hyperbilirubinemia of early life has a hepatic origin. Hollingsworth(6) studied the life span of red cells in newborns at sea level, using radioactive chromium for "tagging" the blood taken at moment of birth, and before its injection into healthy adults. These studies demonstrated that red cells of newborns have a shorter life span than those of adult subjects. In Hollingsworth's opinion(6), this characteristic has no relation to quantity of fetal hemoglobin, degree of macrocytosis, or concentration of hemoglobin at birth; he suggested that the change from oxygen deficiency to normal supply might be a contributing factor. We, therefore, investigated newborns at high altitudes, where the hypoxic condition of intra-uterine life is not replaced, at birth, by normal oxygen pressure.

Methods. Two groups of 5 newborns were studied, one at Oroya, 3,730 meters (12,240 feet) and the other at sea level. About 15 cc of blood were obtained from umbilical cord at moment of birth, and placed in sterile flasks with a small quantity of heparin solution. After incubation at room temperature, with approximately 100 μc of Cr-51 in the form of $Na^2Cr\text{-}5104$, and washing twice with normal saline, the blood was injected into healthy adults, living at sea level. Fifteen minutes after transfusion, and then once a week thereafter, 4 cc of blood were obtained from recipients and degree of radioactivity measured in scintillation counter (well type). Radioactivity was expressed in percentage, considering the first sample as 100% (15 minutes after injection). All samples from each case were read simultaneously to avoid correction for physical decay of isotope.

Results. The results obtained, expressed in survival curves of the injected "tagged" red cells, are presented in Fig. 1 and 2, for newborns at sea level and at high altitudes, respectively. For comparative purposes we have also represented, in both diagrams, survival curves obtained in life span of red cells in 7 healthy adults at sea level, using auto-



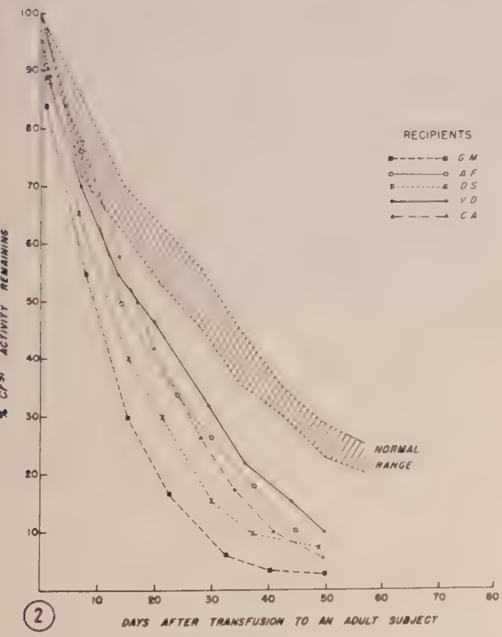


FIG. 1. Newborn red cell survival at sea level.

FIG. 2. Newborn red cell survival at high altitudes.

TABLE I.
Biological Half Life of Cr-51 ''Tagged'' Newborn Red Blood Cells, in 10 Subjects.

	Half life of Cr-51	
	Sea level	High altitude
	12	9
	14	14
	13	12
	11	17
	17	17
Avg	13	14

genous erythrocytes and following a similar technic with Cr^{51}.

As shown by curves in Figs. 1 and 2, red cells from the newborn, at high altitudes and at sea level, were destroyed in a shorter time than red cells from healthy adults. The biologic half life ranged from 11 to 17 days in newborns at sea level, and similar values were observed in newborns at high altitudes (Table I). The biologic half life in red cells of adult subjects at sea level ranged 26 to 32 days, in agreement with observations made by other investigators(6,7,8). Our values for newborns are slightly lower than those obtained by Hollingsworth(6).

Discussion. Evidence obtained in our investigation indicates that red cells of newborns, at sea level and at high altitudes, are destroyed faster than red cells of adult men, when injected into healthy adult subjects. We do not know, however, whether or not the results would be the same if the "tagged" red cells were reinjected into the same infant. If we assume that the same results would be obtained by the 2 experimental procedures, the icterus of the newborn and the decrease in circulating red cells which take place shortly after birth would be based, at least in part, on the accelerated rate of red cell destruction.

The fact that red cells of newborns at high altitudes have the same life span observed in newborns at sea level is not in entire agreement with the supposition that their shorter survival is due to change to an environment having better oxygen supply. At high altitudes there is a prolongation of the hypoxic condition after birth due to lowered ambient pressure, although it is probable that this hypoxia is less severe than the one existing in intra-uterine life.

Summary. The life span of red cells of 5 newborns at high altitudes and 5 newborns at sea level was determined by "tagging" the cells with Cr^{51} and subsequently injecting them

into healthy adults. Survival time of red cells in newborns is shorter than that observed in adult subjects. There was no appreciable difference in results obtained in newborns at sea level and at high altitudes.

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Effect of Pyridoxine in Acute Alcoholic Intoxication. (24591)

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Wordsworth(1) stated that "Vit. B_6 is a specific antidote to alcohol." He based this conclusion on his experience with 6 human cases of acute alcoholic intoxication observed clinically. Small and co-workers(2) made a more extensive and objective investigation of the possible antagonism between alcohol and pyridoxine in patients suffering from acute alcoholic intoxication and in volunteers given varying doses of alcohol. A battery of tests of nervous function was used, and an attempt made to correlate degree of intoxication as appraised by these tests with blood alcohol concentration. The plan of the experiments was to give repeated doses of whiskey until a fairly marked degree of intoxication was demonstrated, then to give pyridoxine in large doses to see whether a sobering effect was produced. In no case was a significant amelioration of the intoxication demonstrated. The authors comment on the difficulty in assessment of a drug for treatment of such a complex of psychic and organic alterations as is comprised in human alcoholic intoxication; they found variations in performance which did not correlate with blood alcohol levels or clinical state of the subjects. It seemed worth while to investigate the problem in an experimental situation more readily controlled than when human drunkenness was involved.

Methods. Over many years we found that degree of alcoholic intoxication in the dog may be quite reliably estimated by the trained observer, utilizing an arbitrary scale of 9 degrees, stage 9 being abolition of corneal reflex, stage 1 slight ataxia on climbing a flight of stairs(3). Seven female dogs varying in weight from 10 to 15 kg received intravenous infusion of 3 g of ethyl alcohol/kg, diluted with physiological salt solution to 15% w/v, in approximately 30 minutes. One hour from start of infusion, and subsequently at hourly intervals for 7 hours, samples of venous blood from a foreleg were analyzed for alcohol(4) and degree of intoxication observed and recorded. These constituted the control runs. The same animals were subsequently subjected to the same procedure, except that they received 100 mg of pyridoxine intravenously just prior to alcohol infusion; while on another occasion 3 dogs received pyridoxine 2 hours after start of infusion. The rate of fall of blood alcohol concentration in mg/100 ml of blood/hour, a reliable criterion of rate of alcohol metabolism, was calculated by subtracting final blood alcohol concentration from that found on first sample and dividing by number of hours elapsed between the 2 samples.

Results. In control runs this averaged 20.6

mg/100 g blood/hour, with pyridoxine 20.5 mg, so that it can be said definitely that pyridoxine under these experimental conditions has no effect on rate of alcohol metabolism.

By plotting degree of intoxication against blood alcohol concentration for each experiment, it was possible by interpolation to estimate degree of intoxication at successive decrements of blood alcohol concentration of 20 mg/100 g. By averaging these values for 7 control runs and 10 runs with pyridoxine it was possible to plot curves of degree of intoxication at different blood alcohol concentrations with and without pyridoxine. From this graph it was apparent that at no blood alcohol concentration was there a difference of more than half a degree of intoxication that could be ascribed to pyridoxine, and where a difference occurred, this was most often a greater degree of intoxication with pyridoxine than without.

Conclusion. In the dog pyridoxine is ineffective in either increasing rate of metabolism of alcohol or reducing degree of intoxication at a given blood alcohol concentration. This is confirmatory of previous work in man, and is conclusive evidence of the uselessness of pyridoxine as an antagonist of alcoholic intoxication.

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Localization of I^{131} Labeled Antibody to Rat Fibrin in Transplantable Rat Lymphosarcoma.* (24592)

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It was possible to prepare an antibody obtained from antisera to the transplantable Murphy-Sturm rat lymphosarcoma that, after labeling with I^{131} and intravenous injection into tumor bearing rats, will localize with a high degree of preferentiality in this tumor (1,2,3). Our recent work indicated that such labeled antibody prepared against this tumor grown in rats, showed a very low degree of tumor localization when injected intravenously into hamsters bearing this tumor as a cheek pouch transplant(3). Also *in vitro* tests indicated that the hamster-grown tumor showed a weak immunological reaction with antibody prepared against the rat-grown tumor. These results combine to suggest that an important antigen in the rat-grown tumor responsible for *in vivo* antibody localization might be some substance produced or deposited by the rat host rather than some antigen intrinsic to the tumor cell itself. Day, Pressman, and associates have recently reported (personal communication) and we have confirmed that *in vivo* localizing antibody isolated from Murphy tumor antisera shows a strong tendency to react immunologically with the rat fibrinogen-fibrin system(4). This suggests that an important antigen responsible for localization of antibody in the rat Murphy tumor might be fibrinogen or fibrin deposited in the tumor by the host, possibly in response to an inflammatory reaction by host tissues to tumor growth. We have therefore immunized rabbits with rat fibrin, clotted the rabbit antisera with normal rat plasma, and from the washed clot isolated and labeled with I^{131} a substance, presumably an antibody, that shows a strong preferential tendency to localize in the Murphy-Sturm lymphosarcoma.

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Methods. Rabbits were immunized against rat fibrin by intramuscular injections of this antigen together with the complete Freund adjuvant once a week for 4 weeks, and pooled antiserum obtained by combining sera from bleedings 10 and 14 days after the last immunizing injection. To a 20 ml portion of such antisera were added 10 ml of pH 8 borate buffer, 16 ml of a saline solution of thrombin containing 40 units/ml, and 8 ml of 2% calcium chloride. The calcium chloride was slightly in excess of that necessary to give free calcium ions after addition of citrated rat plasma. Auxiliary experiments indicate quantities of borate buffer and thrombin are probably not critical. Excess thrombin was added to induce rapid clotting, primarily because of strong inhibition exerted on clotting of rat plasma by this anti-rat fibrin rabbit serum under conditions where rat plasma was not in large excess. Forty ml of rat plasma, containing 16 ml of 2% sodium citrate, were stirred rapidly into the above mixture. A definite but weak clot occurred in about 3 minutes. After 15 minutes at room temperature it was centrifuged 15 minutes at 13,000 rpm in Servall refrigerated angle centrifuge, and the compact fibrin clots homogenized in a mixture of 10 ml of a 3.2% solution of sodium salt of polyglutamic acid and 20 ml borate buffer. The high molecular weight polyglutamic acid was furnished us through the courtesy of Dr. Elkan R. Blout(5) and before use as a reagent in these experiments reacted with free I^{127} iodine to remove sites potentially competing with antibody for I^{131} during subsequent labeling procedures. Other reagents and general procedures have been described(6). The washed fibrin residue obtained by centrifuging and discarding the supernatant fluid was then mixed with 7 ml of 32% urea in polyglutamic acid plus borate buffer to elute into solution antibody to fibrin or fibrinogen present in the washed fibrin residue. Urea has the property of breaking bonds between insoluble antigen and antibody without permanently denaturing antibody(7). After stirring 5 minutes at room temperature and centrifuging at 0°C, the clear supernatant was dialyzed overnight at 0°C against borate buffer to remove urea. The 6.3 ml of dialyzed preparation contained 0.024 mg tyrosine/ml as determined by Lowry *et al.* method modified by omission of the copper reagent(8). This is assumed to correspond to 0.4 mg protein/ml exclusive of polyglutamic acid which gives a zero blank. After storing frozen for a few days a 2 ml portion of this preparation was labeled with I^{131} after preliminary purification by CCl_4 extraction of the radioactive iodine and use of a jet method for mixing on addition of I^{131} as free iodine to protein(6). About 2.6 millicuries of I^{131} were firmly bound to protein; this represented 26% of the starting I^{131}. On the assumption of uniform labeling and of an antibody molecular weight of 160,000, six atoms total iodine were attached to each molecule of antibody. After I^{131} labeling an additional purification separated labeled antibody from I^{131} containing nonspecific material. To the labeled material were added borate buffer, thrombin, and calcium chloride in proportions similar to preiodination purification to a volume of 30 ml. On addition of 10 ml of rat plasma a fibrin clot formed, and the labeled antibody was eluted from the washed fibrin with 32% urea in normal rabbit serum plus borate buffer. Yield of this postiodination purification step was 4%, or 100 μc of I^{131} in the final purified antibody preparation. Labeled antibody from this preparation and 2 similar preparations was injected intravenously into 11 white female rats of Rochester or Carworth Farms strain, both derived from original Wistar stock. Six days before labeled antibody injection these rats had been inoculated by trocar with Murphy-Sturm lymphosarcoma. The rats were sacrificed by saline perfusion about 20 hours after the labeled antibody injection, and the radioactivity of blood, tumor, and other tissues measured using procedures described earlier(6). In other experiments distribution of I^{131} labeled normal gamma globulin from nonimmunized normal rabbits was determined in Murphy tumor bearing animals.

Results. The preparations labeled and purified in this manner, presumably antibody to rat fibrin, show a strong tendency to bind to a rat fibrin clot formed in their presence. When a small amount of such a labeled preparation is added to citrated rat plasma diluted

TABLE I. Distribution of I^{131} in Rats Bearing the Murphy-Sturm Lymphosarcoma the Day Following Intravenous Injection of I^{131} Labeled Antibody Prepared Using Rat Fibrin as Antigen, and of a Control Group Injected Instead with I^{131} Labeled Normal Rabbit Gamma Globulin.

	% dose I^{131}/g tissue	
Organ or tissue	Antibody to rat fibrin, avg values for 11 rats	Normal rabbit γ-globulin, avg values for 15 rats
Blood	3.85 ± .51	4.67 ± .86
Heart	.18 ± .06	.32 ± .07
Lung	.49 ± .18	.77 ± .55
Liver	.27 ± .06	.13 ± .06
Spleen	.55 ± .11	.45 ± .24
Kidney	.22 ± .10	.09 ± .04
Adrenal	.45 ± .18	.26 ± .14
Ovary	.40 ± .12	.70 ± .24
Lymphatic tissue	.30 ± .07	.32 ± .12
Muscle	.07 ± .04	.09 ± .04
Skin	.26 ± .11	.52 ± .18
Small intestine	.19 ± .06	.18 ± .05
Tumor	14.30 ± 3.70	2.16 ± .50

These rats were sacrificed by saline perfusion approximately 20 hr after intrav. inj. of I^{131} labeled antibody or of γ-globulin. Data normalized to 100 g rats. Variability is expressed as stand. dev. of individual animal values.

by a factor of 2 with saline and clotting induced with $CaCl_2$ or thrombin, some 60-80% of the I^{131} label is found in the centrifuged and washed clot. In similar experiments with rabbit plasma only 4-8% of the I^{131} label was carried down in the clot.

The first data column of Table I shows distribution of I^{131} in tumor bearing rats injected with labeled antibody prepared with rat fibrin as antigen. Distribution of I^{131} seemed strain independent in these experiments. The second column shows distribution of I^{131} labeled normal rabbit gamma globulin *in vivo* filtered by the method of McFarlane (9) and Gordon(10). Control values for I^{131} content of tumor obtained by this procedure are higher, measured in percentage of injected dose than after injection of I^{131} labeled untreated normal gamma globulin, and thus give a more conservative estimate of I^{131} accumulation in tumors of rats injected with labeled antibody that may be nonspecific in nature (2). The rats injected with antibody to fibrin averaged 131 g in weight, and tumor weights averaged 1.31 g, or 1% of the rat weight. They contained an average of 14.3% of the injected I^{131} dose. Data on one rat measured by external gamma counting for 3 days after antibody injection indicated I^{131} content of the tumor remained approximately constant while that of blood and viscera decreased so that ratio of radiation dosage to tumor compared with normal body tissues increased with time.

Discussion. The data presented here indicate that an I^{131} labeled antibody to rat fibrin, or to some blood component associated with fibrin during the clotting of plasma, also shows *in vivo* a strong tendency to localize preferentially in the transplantable Murphy-Sturm rat lymphosarcoma. Radioactivity localization in tumor is preferential enough to suggest the possibility of effective radiation therapy of this tumor by this procedure. A possible explanation of antibody localization is that tumor growth induces an inflammatory reaction in the host leading to deposition of fibrin, and in our experiments, subsequent deposition of previously circulating antibody to fibrin. It is also possible that I^{131} labeled antibody combines with circulating fibrinogen, and with fibrinogen conversion and deposition as fibrin the antibody is at the same time deposited in tumor. It appears worthwhile to investigate whether antibody to fibrin will localize in other transplantable or spontaneous tumors. It may turn out that, even in tumors that do not normally induce an inflammatory reaction in the host, preliminary chemotherapy, or radiation therapy not sufficient in itself to destroy the tumor, can induce an inflammatory reaction that can lead to effective radioactivity localization for therapy.

Summary. Rabbits were immunized with rat fibrin and the resulting antisera clotted with normal rat plasma. From the clot a substance was isolated and labeled with I^{131} that showed a strong tendency to bind to rat fibrin clots formed in its presence. This substance, presumably an antibody to rat fibrin, when injected intravenously into rats bearing the transplantable Murphy-Sturm lymphosarcoma localized with a high degree of preferentiality in this tumor.

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Role of Liver and Kidney in Development of Heparin-induced Lipemia Clearing Activity (LCA).*† (24593)

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Since the discovery that injection of heparin endows the plasma with a lipemia clearing activity (LCA)(1,2), it has been an interesting problem to find out which tissues are essential to development or inactivation of this lipolytic enzyme system. Isolated perfusion experiments showed that heparinised plasma develops LCA after it is passed through limbs, thoracic and most abdominal viscera(3-6) but not the brain(5) or the liver(6). Indeed, when plasma already endowed with LCA was perfused through the latter organ is suffered a reduction of that activity(6,9), indicating that the liver inactivates LCA. The present study was undertaken to ascertain the effects of hepatectomy or CCl_4-induced liver damage and of nephrectomy on *in vivo* development of LCA following heparin injection in rats.

Materials and methods. The same experimental plan as in a previous study(7) was employed. Heparin (Connaught's) was injected into the tail veins of control and experimental male Wistar rats weighing 220-280 g, at 0.5 mg/kg. Five minutes after heparin injection 4.5 ml of blood was withdrawn (under ether) from the abdominal aorta of every animal, mixed 1:10 (v/v) with 0.1 molar sodium citrate and centrifuged for 15′ at 3000 rpm. In each instance, 1 ml of the "postheparin" plasma thus obtained was diluted with 1 ml distilled water and mixed in a colorimeter cuvette with 0.5 ml lipemic plasma, produced as previously described(8); turbidity of the postheparin-lipemic plasma mixture was determined immediately and 10′ and 20′ after mixing in a Klett-Summerson colorimeter, against a water blank, using a red filter. Loss of turbidity ("clearing") of the mixtures at any given time was expressed as percentage of initial turbidity at mixing. The animals were injected with heparin and their plasmata obtained and tested for LCA in successive pairs consisting of one control and one experimental, following a precisely timed, staggered schedule. Since the same lipemic plasma batch was used as clearing substrate for each pair of control and experimental plasmata, the results were analyzed statistically with the Null Hypothesis(12). Thus, in every experiment, the mean difference $\pm \epsilon$ between the clearing of every control and its corresponding experimental mixture was computed for each time interval. In tabulating these differences the clearing activities exhibited by the control plasmata were arbitrarily set as references against which those of the experimental plasmata were compared. A difference with a

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TABLE I. Effects of Hemihepatectomy, CCl_4-cirrhosis, Nephrectomy and Thirst on Heparin-induced Lipemia Clearing Activity (LCA).

Group	No. animals per group	Mean body wt change during exp. (g)	Mean clearing activity (expressed as % loss of initial turbidity at mixing) $\pm \epsilon$		Mean difference in clearing activity between postheparin plasma of experimentals and controls $\pm \epsilon$	
			10′ after mixing	20′ after mixing	10′ after mixing	20′ after mixing
1. Hemihepatectomy, 40 hr						
Controls	6	+ 2	20.5 ± 5.5	41.8 ± 6.9		
Hepatectomy	6	− 14	36.4 ± 6.4	58.0 ± 5.2	+15.9 ± 2.1 $(p < .005)$	+16.2 ± 2.8 $(p < .005)$
2. Carbon tetrachloride (0.6 ml of a 50% sol., twice weekly for 6 wk)						
Controls	5	+64	41.4 ± 1.9	67.8 ± 1.5		
Carbon tetrachloride	5	− 1	57.9 ± 1.6	76.6 ± 1.3	+16.5 ± 2.7 $(p < .005)$	+ 8.8 ± 1.9 $(p = .01)$
3. Bilateral nephrectomy, 24 hr						
Controls	8	+ 6	23.4 ± 2.4	42.7 ± 3.2		
Nephrectomy	8	− 2	37.8 ± 2.2	49.6 ± 3.5	+14.4 ± 2.2 $(p < .005)$	+ 6.9 ± 1.8 (p = .01-.005)
4. Thirst, 48 hr						
Controls	5	+17	25.1 ± 2.5	49.5 ± 3.6		
Thirst	5	− 19	22.3 ± 2.2	47.3 ± 3.8	− 2.8 ± 1.2 $(p = .1)$	− 2.2 ± 1.6 $(p > .1)$

positive sign, therefore, implies that experimental plasmata displayed greater LCA than control plasmata, whereas a negative sign indicates the opposite. The following procedures were employed: 1) *Partial hepatectomy*: 2 major lobes of the liver (constituting approximately 50% of the total organ weight) were removed 40 hours before injection of heparin. 2) *Subtotal hepatectomy*: approximately 85% of the liver mass was removed 30 hours before injection of heparin. All hepatectomized rats and their controls were given 5 ml of a 5% dextrose solution s.c. immediately after operation and were offered the same solution to drink *ad lib.* during the post-operative period. 3) *Carbon tetrachloride cirrhosis*: 0.6 ml of a 50% solution of CCl_4 in maize oil was given by stomach tube twice weekly for 6 weeks before heparin administration. All animals become jaundiced at the end of the treatment period and histological studies demonstrated the presence of unequivocal portal cirrhosis in all livers. 4) *Nephrectomy*: both kidneys were removed 24 hours before heparin injection. 5) *Thirst*: to assess the possible role of post-operative hemoconcentration in the results of some of the above experiments, a group of animals was subjected to deprivation of water for 48 hours prior to heparin injection. 6) *Mock-operations*: to ensure comparability between control and experimental animals (where operational trauma was involved) the controls were mock-operated.

Results. Postheparin plasma of the partially hepatectomized, CCl_4-cirrhotic and nephrectomized animals displayed considerably and significantly more LCA than that of the control rats, while thirst had no significant influence on the evolution of this activity (Table I). Since only 2 sub-totally hepatectomized animals survived this drastic operation, no standard error could be computed for their mean value and their data were therefore not included in the table; nevertheless, postheparin plasma of both survivors exhibited considerably greater LCA than that of corresponding controls (averagely +18 at 10′ and +11 at 20′ after mixing).

Discussion. Our findings that removal or damage of the liver and removal of the kidney greatly increase development of LCA in response to heparin constitute indirect evidence that the integrity of the above 2 organs is associated with inhibition or inactivation of the heparin-induced lipolytic activity through some unknown mechanism. In view of the fact that the control animals were mock-operated and that water deprivation did not affect LCA significantly, it is unlikely that surgical trauma or post-operative hemoconcentration were in any non-specific way responsible for the observed marked increments of this activity in the operated rats. Our conclusions concerning the role of the liver are thus in agreement with those of Jeffries(6) and the Spitzers(9) which were based on diminution of the LCA of heparinised or post-heparin plasma after its perfusion through the liver. Our results are also in harmony with the recent clinical findings of increased *in vivo* LCA production in patients suffering from hepatic cirrhosis and chronic alcoholism(10) and the report that liver and kidney extracts inhibited LCA *in vitro*(11).

Summary. 1) Plasma from heparin-injected hepatectomized, CCl_4-cirrhotic and nephrectomized rats exhibited significantly greater lipemia clearing activity than that of heparin-injected normal control animals, when incubated with lipemic plasma *in vitro*. 2) These results represent indirect evidence for the presence of a clearing factor inactivating system in liver and kidney.

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Reticulo-Endothelial Blockade and Capillary Resistance Response to Cortisone and STH.* (24594)

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There have been several investigations of the effect of reticulo-endothelial blockade on ACTH and cortisone induced eosinopenia. Of these, only one concluded that RE blockade diminished eosinopenic response(1). Others denied any difference in hormone induced eosinopenia(2,3,4). One of the latter, however, did note that RE block abolished eosinopenic response to various stresses(2). Kramar and associates showed a marked negative correlation between eosinopenic and capillary resistance responses to exogenous cortisone(5,6); eosinophil count decreasing as capillary resistance rises. These investigators also showed that STH causes a decrease in capillary resistance and this was verified by Wilhelmj *et al.* (7). We used capillary resistance for this investigation because of simplicity of method and because it makes possible the study of effect of RE block on this phase of STH activity.

Materials and methods. Control levels of capillary resistance were established on 14 trained dogs, 7 males and 7 females. Of these,

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TABLE I. Effect of R. E. Block on C.R. Response to Small Doses of Cortisone.

	C.R. (cm Hg)			
Dog	Control*	Cortisone, pre-block*	Cortisone, post-block*	P
I ♂	21 ± 2.20	34 ± 0.25	23 ± 1.41	$>.10$
II ♀	35 ± 2.32	70	35 ± 1.73	
III ♂	21 ± 3.78	70	18 ± 1.97	
IV ♀	14 ± 1.59	26 ± 1.15	16 ± 2.32	$<.05$
V ♂	12 ± 1.71	20 ± 2.12	17 ± 1.61	$<.01$
VI ♀	11 ± 2.47	21 ± 3.39	17 ± 1.53	"
Mean	19 ± 8.23	40 ± 18.5	21 ± 6.90	$>.10$

P values refer to differences between control and post-block values.

* Mean and stand. dev.

6 were given 1 mg of cortisone§/kg/day intramuscularly until capillary resistance stabilized at an elevated level. Injections were discontinued and after capillary resistances returned to control level they were given 1 mg of STH‖/kg/day intramuscularly until they reached a stable lower level. Injections were discontinued and after capillary resistance returned to control level, each dog was given daily intravenous injections of 5 cc India ink¶ diluted with 10 cc of 0.9% saline (1 unit) until total of 1 unit/2.5 kg body weight had been given. Weight of these dogs varied from 8.6 to 14.2 kg. Cortisone and STH regimens were then repeated. To determine duration of block, cortisone regimens were repeated at various intervals. Eight dogs were given 3 mg of cortisone/kg/day for 5 days, then 6 mg of cortisone/kg/day for 4 additional days. They were then given one unit of India ink/day until total of 8 to 10 units had been administered. Each was then given same doses of cortisone for same length of time as given before block. Capillary resistance was determined by negative pressure technic of Kramar and Simay-Kramar(8).

Results. Before blockade all dogs receiving smaller doses of cortisone (1 mg/kg/day) and STH, demonstrated marked rises of CR with cortisone and falls with STH. In every case differences between control and pre-block values showed $P<.01$ (Tables I and II). *After blockade* values after same dose of cortisone were significantly less than before blockade in every dog. When compared to control values, post-block values were significantly elevated statistically in 3 dogs (IV, V, VI) but were unchanged, diminished, or insignificantly elevated in 3 dogs (I, II, III) (Table I). In all 6 dogs, values after STH were not significantly lower than control values (Table II).

All 8 dogs receiving high doses of cortisone, 3 mg/kg/day for 5 days and 6 mg/kg/day for additional 4 days, showed a similar pattern. The C. R. rose markedly before India ink was given. After India ink it rose to level significantly below the pre-block value but significantly higher than control value (Table III).

TABLE II. Effect of R. E. Block on C.R. Response to STH.

	C.R. (cm Hg)			
Dog	Control*	STH, pre-block*	STH, post-block*	P
I ♂	21 ± 2.20	12 ± 2.60	23 ± 2.02	
II ♀	35 ± 2.32	21 ± 2.00	33 ± 2.32	$>.10$
III ♂	21 ± 3.78	10 ± 1.83	20 ± 2.34	"
IV ♀	14 ± 1.59	6 ± 1.15	13 ± 1.86	"
V ♂	12 ± 1.71	8 ± 0.89	15 ± 2.14	
VI ♀	11 ± 2.47	6 ± 1.10	14 ± 2.48	
Mean	19 ± 8.23	10 ± 5.45	20 ± 7.28	"

P values refer to differences between control and post-block values.

* Mean and stand. dev.

The time after block that dogs showed diminished responses to small doses of cortisone varied from less than 43 to more than 107 days.

TABLE III. Effect of R. E. Block on C. R. Response to Large Doses of Cortisone. Differences B minus A, B minus C, and C minus A have probability of $<.01$ for each dog and for the group.

	Capillary resistance (cm Hg)		
Wt, kg	Control (A)*	Cortisone, pre-R.E. block (B)*	Cortisone, post-R.E. block (C)*
♂ 12.3	9 ± 1.2	22 ± 1.5	14 ± 1.0
♂ 10.4	10 ± 0.9	25 ± 1.2	16 ± 1.1
♀ 12.4	11 ± 1.8	28 ± 1.2	16 ± 1.4
♀ 9.9	11 ± 1.3	24 ± 1.7	18 ± 1.1
♀ 13.	7 ± 2.3	19 ± 1.8	14 ± 0.9
♀ 6.2	16 ± 1.3	25 ± 1.7	19 ± 1.7
♂ 7.3	16 ± 1.4	32 ± 2.2	27 ± 0.8
♂ 10.5	16 ± 1.4	32 ± 2.2	26 ± 1.9
Mean	12 ± 2.6	26 ± 4.6	19 ± 4.9

* Mean and stand. dev.

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§ Schering.

‖ Somatrofin (Beef) Horner Montreal (Lots # GE-5 and C35-42D)

¶ Pelikan (C11/1431A) Gunther-Wagner Hanover, Germany.

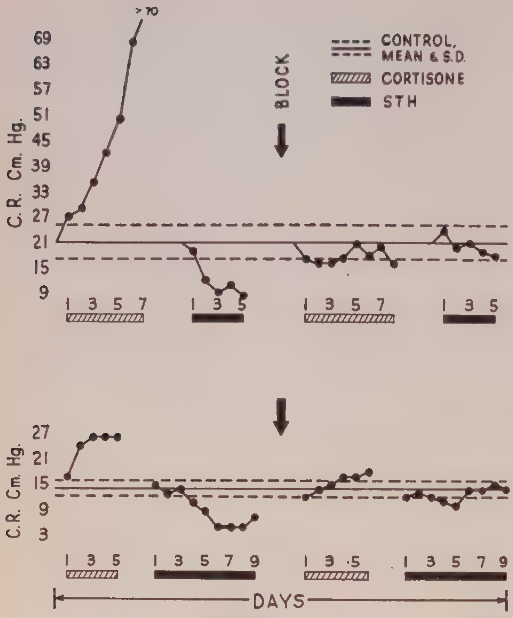

FIG. 1.

Discussion. It has been shown that RE block increases susceptibility of animals to various stresses(9,10,11). With this and the role of corticoids in stress, the work of Kramar *et al.* seems especially interesting. The latter investigators have shown that locally applied cortisone does not increase capillary resistance in the area, unless the animal is stressed(12). They concluded that tissues responsible for capillary resistance become sensitized to cortisone by stress. Results of present experiments suggest that the RE system may be responsible for this sensitization. If this is the case, then the manner in which stress produces its effects may be by causing the RE system to sensitize tissues to glucocorticoids and they, in turn, cause typical tissue and organ response to stress. The manner in which the RE system exerts its effect is not known but the fact that RE block diminishes capillary resistance response to both cortisone and STH suggests that it occurs at tissue level.

Summary. It is shown that blocking the RE system with India ink diminishes or abolishes capillary resistance response to cortisone and STH. That large doses of cortisone can overcome the block suggests that the RE system functions by increasing sensitivity of tissues to cortisone.

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Effect of Leucine-Vasopressin (Phenylalanine-Oxytocin) on Renal Excretion of Na and K in Hydrated Rats and Dogs.[*] (24595)

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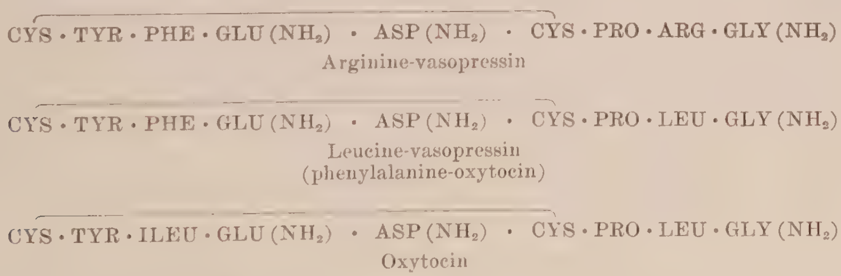
Oxytocin has a powerful naturetic effect in dehydrated[†] dogs(1,2) and rats(3) whereas physiological doses of arginine-vasopressin or lysine-vasopressin have no effect on sodium or potassium excretion in these species. Several analogues of oxytocin have recently been synthesized(4). One of them, the phenylalanine-analogue (du Vigneaud's "oxypressin") is remarkable in having both some antidiuretic and oxytocic activities. This substance which derives its name from having phenylalanine instead of isoleucine in the ring-part of the structure (Fig. 1), and may equally well be called leucine-vasopressin, combines in its molecule the characteristics of vasopressin and oxytocin. It therefore seemed of interest to study its effect on renal excretion of sodium and potassium in hydrated animals.

Methods. Experiments were performed as assays for antidiuretic potency of leucine-vasopressin in dogs and rats, with simultaneous determinations of rates of urinary excretion of sodium and potassium. In dog experiments the method of Ames, Moore and van Dyke(5) using trained, hydrated dogs was employed with the modification that 8% overhydration initially obtained was maintained by administering tap water by stomach tube in 200 ml portions as soon as this volume of urine had been excreted. As index of antidiuretic activity changes in urine osmolality (determined by Fiske osmometer) were used. Assays in rats were done by the method of Thorn(6) with the modification that changes in urine osmolality were used as an index of antidiuretic response. For these determinations the microadaptation unit of the Fiske osmometer was employed. Sodium and potassium concentrations of urine samples were determined by flamephotometry using lithium as an internal standard. A sample of leucine-vasopressin (phenylalanine-analogue of oxytocin) "OTP 79" was kindly supplied by Drs. Berde and Cerletti of Sandoz, Basle. One ml of this solution was stated to contain 1 pressor unit (tested on spinal cat) and 6 antidiuretic units (tested on non-anaesthetized rats by subcutaneous injection). Doses employed were such as should be considered physiological from the vasopressin action (submaximal in antidiuretic effect). All injections were done i.v. through indwelling polyethylene catheters.

Results. Dog Experiments. In 4 experiments with 3 dogs (body weights 13, 15, and 16.5 kg) a total of 16 injections of p-analogue were made. Doses varied from 0.11-0.77 millipressorunits/kg. Results of a typical experiment are illustrated in Fig. 2. Rates of excretion of sodium and potassium rose after each injection. Baseline rates of excretion


CYS · TYR · PHE · GLU (NH_2) · ASP (NH_2) · CYS · PRO · ARG · GLY (NH_2)
Arginine-vasopressin

CYS · TYR · PHE · GLU (NH_2) · ASP (NH_2) · CYS · PRO · LEU · GLY (NH_2)
Leucine-vasopressin
(phenylalanine-oxytocin)

CYS · TYR · ILEU · GLU (NH_2) · ASP (NH_2) · CYS · PRO · LEU · GLY (NH_2)
Oxytocin

FIG. 1. Structures of arginine-vasopressin, leucine-vasopressin and oxytocin.

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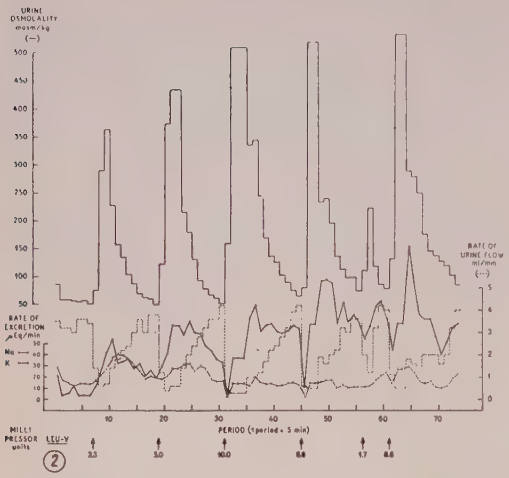
[*] Aided by research grant from Eli Lilly & Co.

[†] In hydrated animals oxytocin is naturetic only if given together with vasopressin.



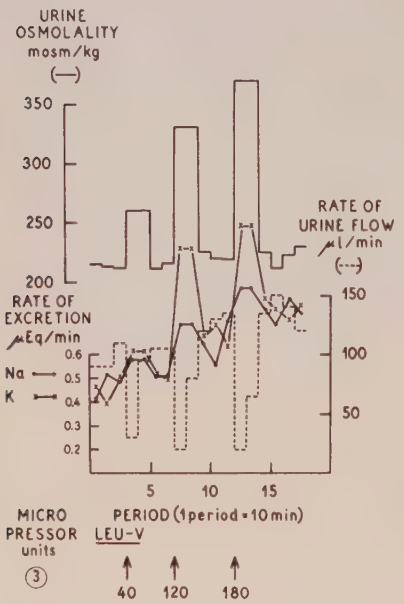


FIG. 2. Effect of leucine-vasopressin (LEU-V) on rate of urine flow, urine osmolality and rates of urinary excretion of sodium and potassium in a hydrated, conscious dog (wt 16.5 kg).

FIG. 3. Effect of leucine-vasopressin (LEU-V) on rate of urine flow, urine osmolality and rates of urinary excretion of sodium and potassium in a hydrated, ethanol anaesthetized rat (wt 250 g).

were 5-70 µeq/min for sodium, 5-15 µeq/min for potassium. Minimum increase in sodium excretion was from 5 to 9 µeq/min after 3.3 mU, in potassium excretion from 4 to 7 µeq/min after 3.3 mU. Maximum increase in sodium excretion (from 20 to 110 µeq/min) and in potassium excretion (from 10 to 60 µeq/min) occurred after injection of 10 mU. A dose-response relation was evident from inspection of the data. The effects on electrolyte excretions were generally of a duration similar to that of the antidiuretic effect.

Rat experiments. In 6 experiments in different rats (average body wt 200 g), a total of 20 injections were made. Doses varied from 10-90 µU/100 g rat. In one experiment 0.5 and 2.5 mU/100 g rat were injected. Results of a typical experiment are shown in Fig. 3. Here also the p-analogue caused an increase in rates of excretion of sodium and potassium. (This for sodium occurred in 16 of the 20 injections, for potassium in 14). Baseline rates of excretion were 0.4-0.7 µeq/min for sodium, 0.3-1.0 µeq/min for potassium. Minimum effect on sodium excretion was a rise from 0.7 to 0.8 µeq/min after 20 µU, on potassium excretion a rise from 0.5 to 0.6 µeq/min after 20 µU. Maximum effect on sodium excretion (from 0.5 to 1.2 µeq/min) was seen after 120 µU, for potassium (1.0 to 2.9 µeq/min) after 5 mU. Also in rats a dose-response relation could be seen. Duration of the effects on electrolyte excretion was roughly similar to that on urine osmolality.

Discussion. The mechanism of action of leucine-vasopressin in increasing the rates of renal excretion of sodium and potassium is obscure. That the mechanism is not one of interference with renal tubular actions of aldosterone or other adrenocortical steroids is suggested by the fact that the rate of excretion of potassium also rose.

It is interesting that leucine-vasopressin, which can be regarded as a structural intermediate between natural vasopressin and oxytocin, given alone has an effect equal to the combined effects of both of these hormones. It appears that a basic amino acid residue in the side chain is essential for strong antidiuretic activity. The leucine residue in the side chain may be of significance for the effect on urinary excretion of sodium and potassium.

Summary. Leucine-vasopressin (phenylalanine-analogue of oxytocin) injected i.v. in submaximal antidiuretic doses to hydrated dogs and rats produced an increase in rate of urinary excretion of both sodium and potassium.

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Pb^{212}(ThB) Tracer Studies in Adult Beagle Dogs.* (24596)

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Interest in metabolism of Pb^{212}(ThB) developed from studies of chronic toxicities of Th^{228}(RdTh) and Ra^{228}(MsTh) in adult beagle dogs. In decay of these isotopes the inert gas thoron (Em^{220}) is formed, and, when decay occurs *in vivo*, a significant fraction of Em^{220} reaches the blood. Part is transported to the lungs and exhaled(1); the remainder decays to give Pb^{212}. Some Pb^{212} leaves the blood, but a significant amount binds to red cells to maintain a high concentration (relative to other activities in blood). For dosimetric purposes we need to know what fraction of Em^{220} decays in blood. This can be determined from measurements of Pb^{212} in blood, if we have a knowledge of Pb^{212} metabolism. There is an abundant literature on lead metabolism, but it relates mainly to chemical toxicity of lead, and includes only a limited number of studies in which carrier-free tracers were used. The affinity of red cells for tracer amounts of lead was reported as early as 1927(2) and the literature includes studies with rats(3) rabbits(4) and humans (5).

Methods. Two healthy young male adult beagle dogs from a large, well controlled colony were used. In Experiment A they received Pb^{212} by intravenous injection; in B, blood was drawn from each dog, tagged with Pb^{212} and reinjected into the dog from which it was drawn. Pb^{212} occurs in the Th^{228} decay chain and can be separated in high purity by de-emanation.

$$Th^{228} \xrightarrow[1.9\ \text{yr}]{\alpha} Ra^{224} \xrightarrow[3.64\ \text{d}]{\alpha} Em^{220} \xrightarrow[54.5\ \text{s}]{\alpha} \xrightarrow{\alpha}$$

$$Pb^{212} \xrightarrow[10.6\ \text{hr}]{\beta,\gamma} Bi^{212} \xrightarrow[60.5\ \text{m}]{\beta,\gamma} \xrightarrow{\alpha} Pb^{208}$$

Air is bubbled through 1 to 2 ml Th^{228} solution in a small culture tube. The inert gas Em^{220} is carried by the effluent air through a short length of capillary tubing into a 1 L. flask. There it decays to Pb^{212} which deposits on the flask walls. As shown by Evans(6), a good yield of Pb^{212} is obtained if volume of the Th^{228} container plus connecting tubing (V_1) is small compared with the receiving flask (V_2), and if the air flow rate is such that V_1 is displaced in a time short compared with the half-period of Em^{220} (54.5 sec), but displacement of V_2 requires several half-periods. To prepare the injection solution for Exp. A, the flask was rinsed with 50 ml citric acid-sodium citrate buffer, pH = 3.5 and total citrate 0.08 M. In B, Em^{220} was bubbled through 10 ml heparinized blood for 1 hour, followed by 30 minutes incubation at 37°C. Since Pb^{212} was determined by gamma measurement, no chemical concentration or ashing of samples was necessary. Blood cell, plasma, and urine samples were measured in a well type liquid scintillation counter; the whole dog counter(7) was used for *in vivo* retention and feces measurements. In both cases sample radioactivity was compared with that of an aliquot of the injection standard. (This procedure automatically corrects for radioactive decay.) Each sample was measured over a period of time sufficient to demonstrate Pb^{212} - Bi^{212} equilibrium.

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* Supported by U. S. Atomic Energy Comm.

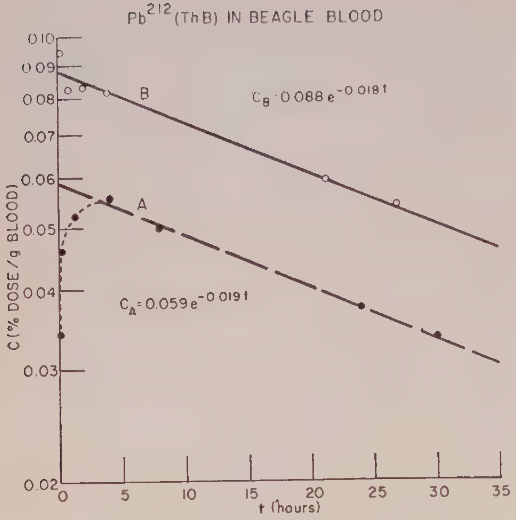

FIG. 1. Pb^{212} concentration in blood following I.V. inj., A, and inj. of tagged cells, B.

TABLE I. Blood Concentration and Excretion of Pb^{212}.

	Dog	Exp. A	Exp. B
		kg	
Dog wt	1	10.6	10.4
	2	9.9	10.0
	Avg	10.2	10.2
		$\mu c\ Pb^{212}$	
Inj. dose	1	15	3.3
	2	15	3.9
	Avg	15	3.6
		% dose/g blood, hr	
Blood conc.	1	$C_A = .064e^{-.022t}$	$C_B = .088e^{-.021t}$
	2	$C_A = .053e^{-.015t}$	$C_B = .088e^{-.015t}$
	Both	$C_A = .0588e^{-.0187t}$	$C_B = .0883e^{-.0194t}$
		hr	
Biological $t_{1/2}$ for Pb^{212} in blood cells*		37.1	37.6
Effective $t_{1/2}$ for Pb^{212} in blood cells*		8.24	8.27
		% dose	
In vivo retention (24 hr)	1	92.1	
	2	90.1	
	Avg	91.1	
Total excretion (24 hr)	1	3.2	
	2	4.4	
	Avg	3.8	
Idem (30 hr)	1	3.8	5.5
	2	4.7	6.2
	Avg	4.3	5.9
Urinary/fecal excretion	1	6.1	3.4
	2	4.5	3.6
	Both	5.1	3.5

* Both dogs.

Results. Blood cells and plasma were measured separately, and in all blood samples 98% or more of the Pb^{212} was in the cellular fraction. Average values of Pb^{212} concentration in blood† during 30 hours after injection (3 Pb^{212} half periods) are shown in Fig. 1. Curve A illustrates the initial rapid rise in concentration and subsequent gradual decrease after intravenous injection of Pb^{212} in citrate solution. Curve B shows the gradual decrease in concentration which follows reinjection of blood which had been tagged with Pb^{212}. (Data were corrected for small amount of Pb^{212} in plasma.) The decreasing portion of curve A parallels curve B, and each can be described by a single exponential (C_A and C_B, Table I). Equations were calculated by method of least square using data for each dog and for both dogs. A biological half period of 37 hours for Pb^{212} in blood cells is calculated from C_A and C_B, which, when combined with the physical half period (10.6 hours), gives an effective half period of 8.2 hours.

Excretion and *in vivo* retention measurements are summarized in Table I. The 24 hour retention value of 91% agrees well with the 96% calculated from excretion. We measured an excess of Bi^{212} over Pb^{212} in urine in amount such that *in vivo* retention is estimated to be 1 to 2% low, while retention determined from excretion is more likely to be high. The two 30 hour excretion values also agree well. Predominance of urinary over fecal excretion is of limited significance, for the half period of Pb^{212} is short compared with lag in fecal excretion. Values (5.1 and 3.5) shown in Table I are therefore not representative of beagle lead excretion in general, and describe only excretion of short lived Pb^{212}.

Discussion. When blood cells are tagged *in vitro* and then reinjected, blood cell Pb^{212} decreases continuously with time, but when Pb^{212} is injected into the blood, Pb^{212} in cells increases to a maximum (which is ⅔ of the tagged cell value at that time), then decreases at the same rate (Fig. 1). To explain this, let us consider blood cells (BC), blood plasma (Pl), and 2 extravascular compartments (EVI and EVII), the second of which includes excretion, which are related as follows:

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† Volume of packed red cells was close to 0.50; hence, Pb^{212} concentration in cells is twice that in blood.

$$\mathrm{BC} \underset{2}{\overset{1}{\leftrightarrows}} \mathrm{Pl} \underset{4}{\overset{3}{\rightleftharpoons}} \mathrm{EVI} \underset{6}{\overset{5}{\rightleftharpoons}} \mathrm{EVII}$$

That blood cells have a much greater affinity for lead than does plasma is demonstrated by the fact that introduction of tracer amounts of lead into whole blood results in 98% or more in the cellular fraction. Schubert and White (3) have shown that the number of cellular binding sites for lead atoms is limited, but that the limit is reached only when mg amounts of lead are used. Immediately after injection of Pb^{212}, concentration in Pl is high and Pb^{212} diffuses into EVI and binds to BC (paths 1 and 3). Depletion of Pl leads to a reversal of the concentration gradient between Pl and EVI very soon after injection, and, since transfer between Pl and EVI is rapid, Pb^{212} returns from EVI to Pl where it is again available to BC, and Pb^{212} in BC continues to increase. At the same time Pb^{212} is also moving from EVI to EVII. This 2-way depletion of EVI continues until the concentration gradient between EVI and Pl reverses again. At the time of this second reversal Pb^{212} in BC has reached its maximum value (65%), it is low in Pl and EVI, and the remaining 35% is mainly in EVII. In Fig. 1, this time corresponds to the maximum in A; thereafter, both A and B represent slow transfer of Pb^{212} from BC to EVII along paths 2, 3, and 5. Since the observed biological half-life for Pb^{212} in cells (37 hours) is long compared with the physical half-life, slow return on path 6 can be neglected (which permits inclusion of excretion in EVII).

From the intercept of B in Fig. 1 and blood specific gravity = 1.06, blood volume is calculated to be 104 ml blood/kg. Parkinson and Dougherty(8) determined red cell and plasma volumes simultaneously, using Cr^{51} tagged red cells and I^{131} tagged human albumin, for 21 normal dogs from the same colony. They found an average blood volume of 94.4 (± 16.6) ml/kg. Our Pb^{212} tagged cell value lies within one standard deviation of their more accurately determined value.

Using the longer lived isotope Pb^{210} Schubert and White(3) found a half period for Pb^{210} in rat blood cells of 30 hours, which is close to our dog value of 37 hours. Following intravenous injection of carrier-free Pb^{210} they report a maximum of 10% in rat blood cells at 25 minutes in contrast with the maximum dog value of 65%.

Hevesy and Nylin(5) used Pb^{212} to determine blood volumes in humans. They report a loss of Pb^{212} from blood cells of less than 4% per hour, from which we calculate 17 hours as a minimum value for the biological half-life of Pb^{212} in human blood cells.

In decay of Pb^{212} to stable Pb^{208} there are 2 beta and one alpha disintegrations plus accompanying gamma radiation. Total energy is 10.1 Mev (8.0 Mev α, 0.83 Mev β, and 1.3 Mev γ). In complete decay of 1 μc Pb^{212} plus daughters, total alpha energy is 2.6 x 10^4 ergs. How is this energy divided among blood, remainder of dog, and excreta?

First, we must consider radioactive equilibrium for we observed an excess of Bi^{212} over Pb^{212} in some urine samples representing 1 to 2% of the total Bi^{212}. In measuring Pb^{212} in blood cells, there is a lapse of about 20 minutes from time blood is drawn to time of the first count. Within this limitation we observed no disequilibrium, and assumption of radioactive equilibrium probably introduces only a small error. Assuming equilibrium, alpha radiation dose to blood for experiments A and B can be calculated using the equations for C_A and C_B (Table I). (In using C_A for Exp. A, we overestimate during the initial period when Pb^{212} in cells is increasing, but calculation shows this error to be only 2 to 3%.) When Pb^{212} is injected intravenously, alpha dose to blood is

$$\frac{8.0 \times 1.6 \times 10^{-6} \times 3.700 \times 10^{4} \times 60 \times 60 \times A}{100} \int_0^\infty 5.88 \times 10^{-4}\, e^{-.0841t}\, dt = .12 \text{ A rads, where}$$

t is in hours and A = μc Pb^{212} injected. The fraction of Pb^{212} atoms which decay in blood is

$$.667 \int_0^\infty e^{-(.0187+.0654)t}\, dt \Big/ \int_0^\infty e^{-.0654t}\, dt =$$

.52. In the tagged cell experiment, alpha dose to blood per μc Pb^{212} is .18 rad, and fraction of Pb^{212} atoms which decay in blood is .78.

At 30 hours Pb^{212} in excreta is about 6% of the total Pb^{212} at that time. Most of the excretion takes place 1 to 3 half periods after injection, so that the actual amount of Pb^{212} which decays outside the dog (*i.e.*, in excreta) is only 1 to 2% of the amount originally injected. Thus our error will be small if we use the simplifying assumption that all Pb^{212} decays inside the dog. Hence, in summary, when blood cells are tagged with Pb^{212} and injected into the dog, about 4/5 of the alpha energy is delivered to blood and 1/5 to the remainder of the dog. When Pb^{212} is injected intravenously, half decays in blood and half in the rest of the dog. It follows then, that when thoron decays in blood, half the resulting Pb^{212} can be expected to decay in blood and half elsewhere in the dog. Therefore, with information gained from these experiments, we will be able to calculate the amount of thoron which decays in blood if we know Pb^{212} concentration in blood and total blood volume.

Summary. Beagle metabolism of short-lived Pb^{212} has been studied following intravenous injection, and after transfusion of blood cells tagged with Pb^{212} *in vitro*. The latter proved to be a satisfactory method to determine blood volume. When Pb^{212} was given intravenously, *in vivo* tagging of blood cells occurred. A maximum of 65% of the activity in cells was reached at 2 to 3 hours after injection, then Pb^{212} in blood cells decreased with a biological $t_{1/2} = 37$ hours and an effective $t_{1/2} = 8.2$ hours. The same decrease occurred after injection of *in vitro* tagged cells. In both experiments essentially all Pb^{212} decayed inside the dog. Half decayed in blood in the *in vivo* tagging experiment; 4/5 decayed in blood when tagging was done *in vitro*.

The author acknowledges assistance of D. R. Atherton, N. Keller, and R. L. Floyd.

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Isolation of Asian Virus from Extrapulmonary Tissues in Fatal Human Influenza.[*][†] (24597)

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Thirty-three influenza-associated deaths were observed in greater Cleveland during the 1957 pandemic of Asian influenza. Asian virus was isolated from lung or trachea in 25 of the 33, and from extrapulmonary tissues in 3 of the 25 respiratory tract-positive cases. Al-

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though influenza virus has been recovered from blood and extrapulmonary organs in experimental infection in animals, occurrence of viremia has not been shown in natural influenza infection in man(1). This report deals with viral studies on pulmonary and extrapulmonary tisues from fatal human cases. Recovery of virus from liver, spleen, kidney, heart, tonsil and lymph node supports the concept that viremia does occur during overwhelming human infection. Detailed analyses of clinical, bacteriologic and anatomic findings of these studies will be reported elsewhere(2).

Methods. Source of material. Tissues were obtained from 33 influenza-associated deaths, 25 of which met the following criteria: 1) history of influenza-like illness; 2) characteristic anatomic findings (acute hemorrhagic and necrotic tracheobronchitis; pulmonary edema and congestion; and intra-alveolar hemorrhage with or without pneumonitis); and 3) isolation of influenza virus from lung or trachea. Extrapulmonary tissues were available in 15 of 25 respiratory tract-positive cases. Tracheobronchial lymph nodes and tonsils were not collected regularly and therefore were tested in only 2 instances. Necropsies were performed within 12 hours after death. *Viral studies.* Organ samples were collected as soon as body cavities were opened and before systematic examination of viscera was carried out. Using washed, flamed instruments, tissues were removed, placed in sterile lusteroid containers, and either tested immediately to establish presence of virus or stored at –70°C until processing. Lung and trachea were examined initially and, when positive, the extrapulmonary tissues were tested. Other organs from respiratory tract-negative cases were not studied. Tracheal mucous membrane was stripped from underlying structures, ground with alundum®, and 10% suspension in veal infusion broth approximated. Twenty % suspensions by weight of lung and extrapulmonary tissues were prepared in similar manner. To each ml of suspension were added 200 units of penicillin and 0.2 mg streptomycin. One-tenth ml was inoculated into the amniotic cavity of each of six 11-day-old embryonated hens' eggs. Amniotic fluid was harvested after incubation at 37°C for 3 days and tested for hemagglutination with chicken red cells at room temperature. Each isolate was identified as Asian type by specific antisera. A negative result with lung or trachea was defined as failure to demonstrate hemagglutination after the original and 1 blind passage on 2 consecutive attempts from original material, and with extrapulmonary tissues after the original and 2 blind passages. Each positive isolate obtained from extrapulmonary tissue suspensions was confirmed 3 times by repeating the procedure from the original tissue. Tissue suspensions were assayed for virus content employing 10-fold dilutions (1:10 to 1:10,000,000) of lung and trachea, and 1:5, 1:25, 1:50 and 1:500 dilutions of extrapulmonary organ suspensions. One-tenth ml of each dilution was inoculated into amniotic cavities of six 11-day-old embryonated hens' eggs. Amniotic fluid was harvested after 3 days' incubation at 37°C and tested with chicken red cells at room temperature. When virus was not found on first passage of a given dilution of organ suspensions, 2 blind passages were made in eggs from pooled amniotic fluids of the first passage. The concentration of virus in lung and trachea is expressed as the reciprocal of highest dilution of suspension from which virus was isolated after first amniotic passage, and in extrapulmonary tissues, after first and subsequent passages.

Results. Asian strain influenza virus was isolated from extrapulmonary organs in 3 of 15 cases: in one it was present only in the tonsil; in the second, in spleen and lymph node and in the third, in liver, spleen, kidney and heart. The clinical, anatomic and bacteriologic findings in the 3 cases are shown in Table I. The 2 children were previously healthy, but the adult had serious pre-existing liver and kidney disease.

The histologic appearance of virus-positive extrapulmonary tissues (Table II) revealed acute nonspecific inflammatory changes in addition to pre-existing chronic disease. Although virus was isolated from heart muscle (Case 19) showing acute arteriolar lesions, virus could not be demonstrated in myocardial suspensions from 13 respiratory tract-positive cases, 3 of which exhibited acute myo-

TABLE I. Clinical, Anatomic and Bacteriologic Findings.

Case No.	Age (yr)	Sex	Race	Duration illness (days)	Clinical features	Postmortem findings	Bacteriology	
							Lung	Trachea
6	10	♂	Non-white	2	Sudden onset of influenza-like illness; 7 siblings simultaneously ill; developed bilateral pneumonia	Pulmonary edema, congestion and hemorrhage; Focal pneumonitis; Acute necrotizing tracheobronchitis; Acute nonspecific changes in splenic and lymphoid follicles; Cerebral edema and congestion; Pseudotubular change in adrenals; No pre-existing disease	Hem. staph.	Hem. staph.
19	59	♀	White	4	Sudden onset of influenza-like illness; found dead at home; chronic alcoholic with liver cirrhosis	Pulmonary edema, congestion and hemorrhage; Acute necrotizing tracheobronchitis; Fibrinoid degeneration of myocardial arterioles; Portal cirrhosis; Nephrosclerosis; Chronic pyelonephritis	Sterile	Sterile
28	4	♀	”	4	Coryza treated by parent with penicillin; found dead at home	Pulmonary edema, congestion and hemorrhage; Focal pneumonitis; Acute necrotizing tracheobronchitis; Acute myocarditis; Acute follicular tonsillitis; Necrosis of splenic and lymphoid follicles; No pre-existing disease	”	Hem. staph.

carditis histologically, nor in brain suspensions from 13 cases, 2 of which were characterized clinically by widespread involvement of the central nervous system. Virus titrations were performed on tissues from both extrapulmonary-positive group and 3 other cases from which virus had been recovered only from the respiratory tract (Table III). Concentration of virus, expressed as reciprocal of highest dilution of suspension from which virus was isolated, ranged in pulmonary tissues from 100 to 100,000 and in extrapulmonary tissues from 25 to 500 after the first amniotic passage. After one or 2 blind passages of those dilutions of organ suspensions negative on first passage, virus was detected in the 1:5 dilution of spleen in Case 19, the 1:500 dilution of spleen in Case 6, and the 1:1000 dilution of tonsil in Case 28.

TABLE II. Histologic Appearance of Virus-Positive Tissues.

Case No.	Organ	Histologic lesion
6	Spleen	Nonspecific inflammatory reaction
	Lymph node	*Idem*
19	Liver	Portal cirrhosis
	Spleen	Nonspecific inflammatory reaction
	Kidney	Nephrosclerosis; chronic pyelonephritis
	Heart	Fibrinoid degeneration of arterioles with focal fibrin thrombi
28	Tonsil	Acute follicular tonsillitis; necrosis of follicles

Discussion. Hamre and her co-workers(1) demonstrated virus in blood and extrapulmonary tissues in experimental influenza infection in animals, and suggested that viremia might also occur in natural influenza in man. Attempts to isolate virus from blood were not carried out in our study, but virus was obtained from extrapulmonary tissues. Localization of virus in distant organs lends strong support to the presence of viremia during overwhelming human infection.

Flewett and Hoult(3) have recently de-

TABLE III. Concentration of Virus in Tissues from Cases of Human Influenza.*

Organ	No. positive/No. tested†	Case No.					
		6	19	28	4	16	29
Lung	19/32	1,000	1,000	0	100	1,000	100,000
Trachea	22/29	100	100,000	100,000	10,000	100	NT
Liver	1/14	0	50	0	0	0	"
Spleen	2/14	25 (500)‡	0 (5)§	0	0	0	"
Kidney	1/13	0	25	0	0	0	"
Heart	1/14	0	50	0	0	0	"
Lymph node	1/ 2	25	NT	NT	NT	NT	"
Tonsil	1/ 2	NT	"	500 (1,000)§	"	"	"

* Conc. = reciprocal of highest dilution of suspension from which virus was isolated after first amniotic passage.

† One or more extrapulmonary tissues were available for testing from 15 cases.

‡ Reciprocal of highest dilution after third passage.

§ *Idem* second .

0 = Negative. NT = Not tested.

scribed fatal cases of Asian strain influenza associated with encephalopathy at height of acute attack. Virus was isolated from both brain suspension and a pool of lung and trachea in 1 case. A 1:10 dilution of brain suspension failed to yield virus, although virus grew well in eggs inoculated with a 1:1000 dilution of lung and trachea. The authors suggest their findings may have been due to postmortem room viral contamination of brain samples, or to viremia.

Despite the relatively low concentration of virus in extrapulmonary tissues in our study, it is unlikely that the findings are the result of contamination either in autopsy room or laboratory. Collection of specimens was carried out before the pathologist conducted routine dissection. Instruments were washed and flamed immediately prior to use. Samples of tissue were placed in sterile lusteroid containers as soon as they were removed from body. Positive isolation of virus from extrapulmonary tissues was repeated at least 3 times for each sample tested. Finally, systemic manifestations, both clinical and anatomic, suggest the likelihood of blood-stream dissemination of virus in fatal influenza in man.

Summary. Asian strain virus was demonstrated in extrapulmonary tissues from 3 influenza-associated deaths. This finding suggests the occurrence of viremia in overwhelming influenza in man.

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Effect of Filtration Procedures on Immunogenic Activity and Safety of Formalinized Poliomyelitis Vaccine (Salk Type). (24598)

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Methods for production of formalinized poliomyelitis vaccine on a semi-industrial scale have been described by us(1). Filtration of virus pools before formaldehyde-inactivation through fritted glass filters proved inadequate in production of safe vaccine pools; Seitz filtration of virus suspensions before inactivation was found to be essential for preparation of non-infectious vaccine. In preparation of vaccine pools, we followed Minimum Requirements of U.S. Public Health Service(2) in adding a 2nd Seitz filtration during formaldehyde-inactivation process. We felt, however, that this filtration is deleterious to the antigen and reduces significantly the immunogenic activity of pools treated in this way. To test the effect of filtrations during inactivation process, experiments were carried out to replace or omit the 2nd Seitz filtration. The results of these experiments and their effect on safety and antigenicity of poliomyelitis vaccines are here reported.

Materials and methods. Preparation of tissue cultures and virus suspensions, and safety and potency testing of monovalent pools were carried out according to methods described earlier(1). Changes were introduced in processing and in inactivation of some monovalent pools, as follows: 1. *Filtration of crude live virus suspensions before inactivation.* Two changes were introduced in these filtrations: a) Replacement of Corning fritted glass tubular filters, porosities Medium to Ultrafine, by Seitz clarifying pads, grades K3 and K7 (Seitz Werke, Germany). The filter used was Perfectum filter, model LF 280T (Popper & Sons, U.S.A.) which allows simultaneous filtration through 2 filter pads of 14 cm diameter. The Perfectum filter was adapted for continuous filtration by removal of pressure gauge and addition of stainless steel baffle to avoid rupture of pad by the incoming fluid. Virus suspension was introduced continuously into inlet tube of sterile filter. Sterile air under pressure of 0.3 atm. was applied to the carboy which served as feeding vessel. Filtration through Seitz clarifying pads was preceded by filtration through cotton filter and tubular filter, porosity coarse, and followed by filtration through Seitz sterilizing (STl) pad. b) Washing procedure for Seitz sterilizing pads as well as clarifying pads was altered; instead of Hanks solution, Medium 199 (pH 7.0) was used to wash the pads before passing the virus suspension. 1800 ml of Medium 199 were passed through the pad before filtration of the virus suspension. 2. *Filtration during inactivation process.* a) A number of pools were divided on 4th day of inactivation into 2 approximately equal parts. One part was filtered through washed Seitz sterilizing pad, while the other was passed through Fine-Ultrafine Corning glass tubular filters. Both aliquots were further treated as separate pools; they were inactivated for additional 8 days, and tested for safety and potency separately. b) In several pools filtration on 4th day of inactivation was omitted entirely. 3. *Total time of inactivation* of all monovalent pools was reduced from 14 to 12 days. Large samples, of at least 500 ml, were withdrawn for safety testing on 9th and 12th days of inactivation.

Results. Titers of Seitz filtrates obtained by passing suspensions through Hanks-washed pads are reduced, the loss of virus titer ranging from 0.3 to 0.8 log. Filtrates, passed through Seitz pads washed with M199 prior to filtering, show almost no loss in titer (0.1 to 0.25 log differences being regarded as within experimental error limit of test).

Table I summarizes results in which 4 monovalent pools were each divided, on 4th day of inactivation, into 2 parts, one of which was passed through Seitz filter pad washed with M199, and the other through a Fine-

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* The authors express thanks to Yehoshua Saiar, Rachel Riger, and Hedva Kampf for technical assistance.

TABLE I. Potency of Vaccine Which Had Been Filtered on 4th Day of Inactivation.

			Potency tests*							
			Guinea pig extinction end point†		Guinea pig serum end point‡		Chick extinction end point†		Chick serum end point‡	
Pool No.	Type	Vol (liters) of Seitz filtrate before inactivation	FUF	Seitz	FUF	Seitz	FUF	Seitz	FUF	Seitz
201	I	16	2.6	.9	80	5	.9	.0	21	3
202	I	19	1.8	1.8						
206	I	18	3.2	2.6	517	221			291	313
U	III	16	2.5	.0	9	0	1.1	.0	40	2

* FUF = 2nd filtration carried out through Fine-Ultrafine glass filter. Seitz = 2nd filtration carried out through a washed Seitz filter.

† Extinction end point = $\log_{10}$ of 50% titer of antigen.

‡ Serum end point = mean antibody titer of sera from guinea pigs or chicks inoculated with undiluted vaccine.

Ultrafine Corning tubular glass filter. Safety tests carried out on large samples, ranging 400-700 ml, taken 3 days prior and at end of inactivation period, from Seitz filtered as well as from Fine-Ultrafine glass filtered aliquots, were unequivocal: no live virus was isolated from any of these samples nor from subcultures carried out at one, 2, and 3 week intervals. Results of potency tests showed in 2 instances (pools 201 and U) significant differences in antigenicity. The guinea pig extinction point was 50 and 300 times higher, respectively, for aliquots filtered through Fine-Ultrafine filters than for those filtered through Seitz filters. This difference is also evident from guinea pig serum end point titers, as well as from chick extinction and serum end point titers. In the monovalent Type I pool, 202, no loss of potency seemed to have resulted from filtration through the Seitz filter. The 2 aliquots of pool 206 also showed no marked difference, whether filtered through Seitz or Fine-Ultrafine filter. Thus it appears that filtration through a Seitz filter on 4th day may sometimes affect immunogenic activity of the vaccine. Filtration through a Fine-Ultrafine filter seems to do no harm to the antigenicity of the material.

A number of monovalent pools were left unfiltered during inactivation period and the results of safety and potency tests carried out on such pools are presented in Table II. For comparison, results are given also on safety and potency tests carried out on pools which have undergone Seitz filtration on 4th day of inactivation. Samples of 300-800 ml, withdrawn from all these pools 3 days prior and at the end of inactivation period, contained no live poliovirus. Guinea pig extinction points (expressed in $\log_{10}$ units) were very high for pools in which the second filtration was omitted; they ranged from 2.2 to 3.2, and in most instances were higher than 2.5. Guinea pig serum end point titers ranged from 60 to over 1000. Antigenicity of monovalent pools, which had been filtered through Seitz filter on 4th day of inactivation was much lower, especially for Type I pools.

To strengthen confidence in the absolute safety of pools in which the 2nd Seitz filtration was omitted, additional samples were withdrawn from several pools on 7th day of inactivation (5 days prior to end of inactivation period) and tested for safety in the usual manner. Four such 7th day samples of 300-500 ml each were tested and found free of live poliovirus. These findings, in addition to consistent negative safety tests on the 9 monovalent pools included in Table II, show that omission of 2nd Seitz filtration was not at the expense of safety. After incorporation of these monovalent pools into trivalent vaccines, absence of live poliovirus was further proved by safety tests carried out in tissue culture on large amounts of trivalent vaccines (over 1500 ml), by safety tests in cortisone-treated monkeys, and by use of such vaccines in large numbers of infants and children, many of whom were triply negative for poliomyelitis antibodies at time of vaccination.

Discussion. To ensure absolute safety of formalinized poliomyelitis vaccine, the U. S. Public Health Service Technical Committee on Poliomyelitis Vaccine recommended dou-

TABLE II. Results of Safety and Potency Tests on Monovalent Pools in Which Filtration during Formaldehyde Inactivation Process Was Omitted, as Compared with Lots Filtered through Washed Seitz Pads.

Pool No.	Type	Vol (liters) of Seitz filtrate before inactivation	Filtration on 4th day of inactivation	Results of safety tests* a	b	Guinea pig extinction point	Guinea pig serum end point
200	I	4.3	Omitted	300	750	2.2	70
209	I	18.0		400	500	3.1	60
212	I	13.5			800	>2.5	160
215	I	15.5		300	450	>2.0	
216	I	15.0		500	350	>2.0	
210	II	12.0		600	600	3.2	≥1000
213	II	12.0		400	650	3.1	
208	III	6.0		650	550	>2.5	
211	III	13.0		450	350	2.9	
190	I	19.0	Seitz	350	500	.6	
193	I	17.0		600	700	.9	
195	I	16.0		450	600	1.3	
191	II	16.0		500	500	1.7	
194	II	13.0		550	600	1.4	
197	II	10.0		450	700	2.6	
192	III	14.0		500	550	1.3	
198	III	15.0		450	350	1.8	

* Milliliters tested up to 14 days' subculture and found negative. a = sample taken 3 days before end of inactivation period; b = sample taken at end of inactivation period.

ble Seitz filtration, the first before addition of formalin, and the second during the inactivation process(3). This recommendation was based on experience gained in various laboratories in United States, which showed that the second filtration through Seitz filter pads is much superior to filtration through glass filters in yielding a product free of detectable poliovirus(4). Experience in the field on use of such doubly-filtered vaccines (commercial U.S.A. vaccines) in children proved recently to give poor antibody responses after basic 2-dose immunization especially for Type I and III components(5,6 and personal communications). Immunogenic activity of such vaccines in triply negative children proved to be much inferior to that of the experimental vaccines used in the past by Salk(7).

In our own experience, immunogenic activity of doubly-filtered vaccines was low, especially for Types I and III. Guinea pig extinction points (expressed in $\log_{10}$ units) of such doubly filtered pools ranged from 0.6 to 1.3 for Type I, 1.4 to 2.6 for Type II, and 1.3 to 1.8 for Type III poliovirus. Infants and children, receiving 2 basic inoculations of 0.5 to 1.0 ml each of such vaccines, showed conversion in only 10 to 20%. The evidence suggested that Seitz filtration during inactivation process removed large amounts of antigen (especially Types I and III). The present report confirms this assumption. Monovalent pools in which the 2nd Seitz filtration was either omitted or replaced by filtration through a Fine-Ultrafine filter, proved to be of high antigenicity in guinea pigs and also in triply negative infants and children (our unpublished results). The safety of such treated pools was shown in our data as unequivocal, all pools tested were free of live poliovirus. However, whether omission of a 2nd Seitz filtration can consistently result in safe vaccines needs further confirmation on a larger number of pools, and possibly on larger volumes of vaccine.

Summary. Seitz filtration of poliomyelitis vaccine pools during formaldehyde-inactivation process was found to lower their immunogenic activity. Monovalent vaccine pools in which filtration through Seitz filter pads during inactivation process was either omitted or replaced by filtration through fritted glass filters, proved to be of high antigenicity both in guinea pigs and in triply negative children. Routine safety tests in tissue culture carried out on such pools were consistently negative.

Additional proof of the safety of such pools was provided by absence of live poliovirus from large samples withdrawn as much as 5 days prior to end of inactivation period, and also by use of these vaccines in the field.

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Inhibition of Pepsin and Lysozyme by Acidic Polymers (24599)

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A series of acidic macromolecules arising from polymerization of phenols, or phenolic carboxylic or sulfonic acids have been shown to inhibit ribonuclease and other hydrolytic enzymes(1). Certain members of the series likewise showed activity in suppressing formation of gastric ulcers in Shay rats.* We were interested to learn if this biological activity could be correlated with inhibitory power against pepsin and/or lysozyme. Levey and Sheinfeld(2) and Marini and Levey(3) reported that chondroitin sulfuric acid inhibits pepsin *in vitro*, and that this polymeric acid also reduces the number of ulcers formed in the Shay rat stomach. Whereas pepsin has long been implicated in the etiology of gastric ulcers(4) the participation of lysozyme was suggested by K. Meyer *et al.*(5) and has been doubted by Reifenstein *et al.*(6), who feel that the increased tissue lysozyme found in the margins of active, but not of chronic, gastric ulcers represents a tissue response to the lesions rather than an etiological factor. Reports on inhibition of lysozyme action by acidic polymers and related compounds have appeared from several laboratories; the substances include hyaluronic acid(7), treburon and heparin(8,9) alkyl sulfates, fatty acids and alcohols(5,10,11). *Polymers.* The 4 polymers discussed in this paper were prepared as described(1) by condensation of I. Formaldehyde and hydroquinone sulfonic acid. II. Formaldehyde and 3,4-dihydroxybenzene sulfonic acid. III. Furfural and hydroquinone sulfonic acid. IV. Acetaldehyde and hydroquinone sulfonic acid.

Methods. Measurement of lysozyme inhibition. The criterion most widely used to evaluate lysozyme activity, *viz.*, clearing of a suspension of *M. lysodeicticus*(12,13,14,15) probably is not reliable(16) when the reaction is allowed to proceed at pH2 followed by alkalinization(17). Optical methods appeared unattractive for study of inhibition by colored substances. Enzyme activity was measured by viscosimetry according to Meyer and Hahnel(18). The substrate (80 mg) was dissolved in 30 ml of McIlvaine's buffer (pH 2.15) containing 0.2 M sodium chloride. A small amount of gummy residue was removed by centrifugation after the solution had been agitated for one hour. The solution may be kept for at least 8 hours at room temperature without change in viscosity. Twice recrystallized lysozyme (Worthington Biochemical Corp.) was dissolved in 0.9% sodium chloride to give 500 μg/ml. The polyphenolsulfonic acids used as inhibitors were dissolved in distilled water. Viscosimetry was patterned after the procedure used by Northrop for measurement of peptic activity(19). Four-ml aliquots of substrate solution were placed into 50-ml test tubes. To each tube, 0.5 ml

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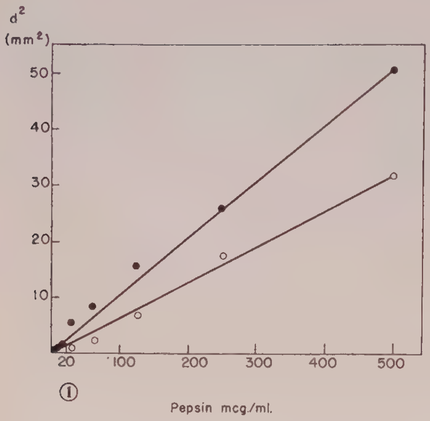
* Dr. A. Plummer of this Department observed the anti-ulcer effect in Shay rats. We thank Dr. Plummer for permission to refer to his results.



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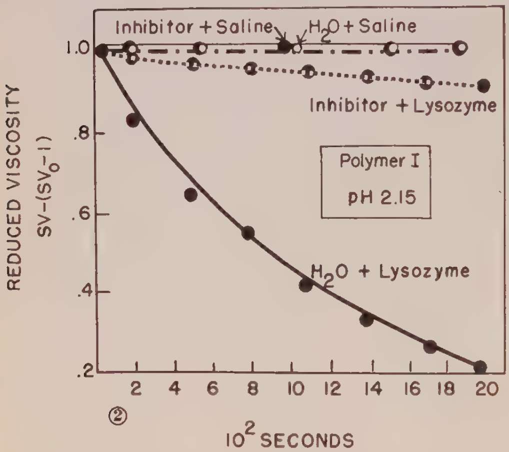

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FIG. 1. Plot of square of distance of albumin digested (d^2) in Mett tubes during 16 hr at 37° by pepsin in 0.056 N HCl in presence (○) and absence (●) of 200 μg/ml of polymer I.

FIG. 2. Inhibition of lysozyme (50 μg/ml) action by 240 μg/ml of polymer I at pH 2.15.

of water or inhibitor solution was added and the mixture was well stirred. For enzymatic digestion a tube was equilibrated in a water bath at 35.5° ± 0.04. At zero time 0.5 ml of enzyme solution was rapidly mixed with the substrate solution, and the digestion mixture was introduced into an Ostwald viscosimeter submerged in the water bath. Total elapsed time as well as outflow time were recorded for each viscosity measurement. For measurements at pH 5.25, the optimum for lysozyme activity(18), the same procedure was used with suitably reduced concentrations (Fig. 3 and Table I) of enzyme and inhibitors. However, at pH 5.28 and 35.5° the substrate undergoes a slow non-enzymatic decrease in viscosity, correction for which was made accordingly. *Measurement of pepsin inhibition.* Formol titration of gelatine-pepsin digests, viscosimetry of edestine-pepsin solutions, and determination of non-protein nitrogen were not suitable to measure pepsin activity in the presence of the polymers under study. Only the solubilization by pepsin of coagulated egg white allowed us to compare, in an approximate fashion, the inhibition exerted by 3 of the polymers. Mett tubes were prepared from the liquid portion of egg white by coagulation in pyrex melting point capillaries as in the standard procedure(20). Twice crystallized pepsin (Worthington Biochemical Corp.) was used. Sections of the albumin tubes were dropped into solutions of enzyme with and without inhibitor and incubated at 37°. Degree of digestion was estimated by measuring, on a vernier microscope stage, the length of albumin column dissolved at each end of the tube sections. Fig. 1 shows that under the conditions chosen the proteolytic power of serial dilutions of pepsin in presence or absence of a fixed amount of inhibitor is proportional to the square of the length of the albumin column digested.

Results. (A) Viscosimetry. If t and t_{H_2O} are the outflow times in seconds, of solution and solvent, respectively, and SV is the specific viscosity, then

$$SV = \frac{t}{t_{H_2O}} - 1. \qquad (1)$$

Initial specific viscosity SV_0 was determined by extrapolation to zero time from time-viscosity graphs.

To facilitate comparison of the plots of different runs, the quantity (SV_0-1) was subtracted from each of the SV-values found in a run, so that all curves begin at 1.00 on the viscosity axis. The viscosity values so obtained were plotted against time, assigning to each point a time T equal to elapsed time plus one-half the outflow time measured. T = elapsed time + ½ t. Results are represented in Figs. 2 and 3. Initial rates were calculated approximately as per cent viscosity change per unit time occurring during the first 500 seconds.

TABLE I. Inhibition of Lysozyme (50 μg/ml) at pH 2.15.

Reaction mixture	t_o	t_{500}	Rate (% viscosity change per sec.)	Inhibition, %
Uninhibited	126.8	103.7	.073	91
Polymer I, 240 μg/ml	133.2	131.1	.0064	
Uninhibited	122.6	98.3	.078	92
Polymer II, 250 μg/ml	125.0	123.0	.0064	
Uninhibited	125.2	107.1	.056	92
Polymer III, 240 μg/ml	132.0	130.5	.0046	
Inhibition of lysozyme (.2 μg/ml) at pH 5.28				
(1) Substrate + saline	125.6	125.1	.0016	
(2) Substrate + polymer I (1.9 μg/ml) + saline	125.6	124.5	.0034	
(3) Uninhibited	124.4	106.4	.0580	
(4) Polymer I (.48 μg/ml)	132.4	119.5	.0390	37*
(5) " (1.9 μg/ml)	132.6	125.0	.0230	65*
(1) Same as (1) above				
(2) Substrate + polymer III (1.9 μg/ml) + saline	155.0	154.5	.0012	
(3) Uninhibited	126.6	117.2	.0266	
(4) Polymer III (1.9 μg/ml)	137.6	131.1	.0190	38*

* Rates of enzymatic reactions were corrected by subtraction of spontaneous (non-enzymatic) viscosity change, before % inhibition was computed.

$$\text{Rate} = \% \text{ change in SV/second} = \frac{(t_o - t_T) \cdot 100}{(t_o - t_{H_2O}) \cdot 500}$$

where t_T and t_o are the outflow intervals at time T and zero, respectively. The values for t_T and t_o are obtained by means of equation (1) from the SV-values taken by interpolation and extrapolation, respectively, from the specific viscosity-time plots. The rates allow computation of per cent inhibition values, which are presented in Table I.

Scarcity of substrate made necessary the following reasonable assumptions. (a) The substrate does not undergo a viscosity change at pH 2.15 in presence of polymers II or III, although this was demonstrated only for polymer I. (b) The spontaneous viscosity decrease at pH 5.3 determined in presence of polymer I is applicable to polymer III. (c) The effect of inhibitor concentrations on rate of spontaneous (non-enzymatic) viscosity decrease is negligible.

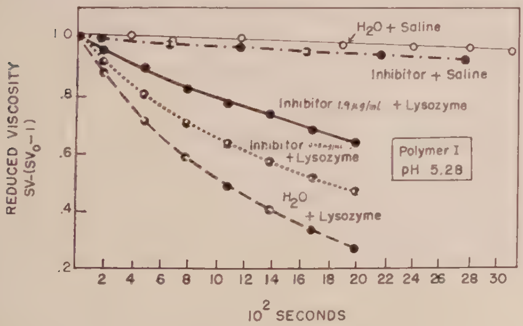

FIG. 3. Inhibition of lysozyme (0.2 μg/ml) action by polymer I at pH 5.28.

(B) *Peptic digestion.* Results of the pepsin experiments appear in Table II.

Discussion. Since only polymer I gave a positive effect in Shay rats the all-or-nothing effect noted in the animal experiments is not reflected by a similar, sharp delineation of anti-enzyme potencies: each of the polymers tested inhibited one, or both, of the enzymes. However, at the 250 mg level inhibition of pepsin caused by polymers II and IV is only about 50%, compared to 80% for polymer I. No difference was seen among the 3 polymers tested at pH 2 against lysozyme but at pH 5.2 again polymer I gives greater inhibition than polymer III. Conceivably such a difference might also have been observed at pH 2 if lower inhibitor concentrations were employed, but exhaustion of the substrate prevented us from making such measurements.

Thus, the *in vivo* experiments can be related to at least a trend in the anti-enzyme behavior of the compounds. If the inhibitory power of the polymers with regard to pepsin and/or lysozyme is, indeed, connected with their anti-ulcer potency, then the relatively small differences observed in the present study

TABLE II. Inhibition of Peptic Digestion of Coagulated Egg White in Mett Tubes. Pepsin: 250 μg/ml in 0.056 N HCl. Incubation: 16-20 hr at 37°. d = length of egg white column digested.

		Polymer I			Polymer II			Polymer IV		
Inhibitor, μg/ml	None	500	250	125	500	250	125	500	250	125
d^2	2450	121	529	1764	169	1296	1681	121	1089	1521
	2500	121	676		1369	1369	2116	121	1156	1849
	1764	100	289	1190	812	812	1296	144	900	1521
	2304	121	504	1480						
% inhibition avg	0	95	81	32	95	49	24	94	53	27

might lead one to search for more powerful anti-pepsins, or anti-lysozymes in the hope of uncovering therapeutically more valuable compounds.

Summary. (1) Ability of 4 acidic polymers to inhibit lysozyme and/or pepsin has been measured. (2) Their anti-enzyme effect is compared to their action in the Shay rat.

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Force-Velocity Relationship of Tetanus Toxin Treated Skeletal Muscle.* (24600)

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The mechanical properties of muscles actively shortened by tetanus toxin injection vary from normal in a manner and to a degree not solely attributable to atrophy(1). These muscles exhibit increased tetanus-twitch ratio, shortened twitch relaxation time, as well as lower fusion frequency(2), and an augmented semi-dynamic stiffness, at short degrees of stretch, following bouts of tetanic stimulation(3). Such observations are not readily interpreted when considered as isolated facets of the mechanical behavior of such actively shortened muscle. To extend

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* Aided by grant from Muscular Dystrophy Assn. of America.

the interpretation of prior observations, an investigation of force-velocity relationship of these preparations was made. The theoretical and practical importance of this relationship has been stated by Wilkie(4). Energy released during shortening of muscle appears to govern the shape of the force-velocity curve. The constants *a* and *b* of Hill's(5) characteristic equation $(P + a)v = (P_o - P)b$ where *a* is the 'extra' heat/cm of shortening, and *b* is a constant with velocity dimensions can be derived equally well from studies of force and velocity, or of heat and length.

Methods. Thirteen male albino rats averaging 394 g in body weight (range 329-428) were used for the study. Active shortening of one gastrocnemius muscle of each animal was induced by unilateral injection of tetanus toxin into the popliteal space on that side. As a routine procedure, right and left muscles alternately were selected as experimental preparations. Maximum hyperextension of the limb without overt spread to the contralateral side was assured by careful adjustment of the toxin dosage. Gastrocnemii were exposed under chloralose anesthesia (15 mg/100 g body weight) 14 days after toxin injection(3). After measurement of the *in situ* resting length and severance of the insertion so as to include a portion of the os calcis, an 11 mm wound clip with attached nylon monofilament was fastened to the free tendinous end. The monofilament was connected to an isotonic myograph employing an electromechanical transducer(6). Muscle shortening produced a potential change which was registered on a Dumont 322A cathode ray oscilloscope as a trace that was preserved on 35 mm film. Maximum initial velocities were calculated from enlargements of these photographs. Stimulation of the preparation consisted of brief slightly supramaximal square wave pulses, frequency 105/sec., applied to the muscle through fine, sharply pointed silver wire electrodes. Four-minute intervals of rest were allowed between successive tetani. Subsequent to the testing procedures all muscles were carefully excised and their wet weights determined. Plots of $(P_o - P)/v$ against P, as described by Katz (7) were used to calculate the constants *a* and *b* of Hill's equation(5). The small sample *t* test was utilized in statistical analyses. Probabilities of 0.01 were accepted as evidence for a true difference in mean values.

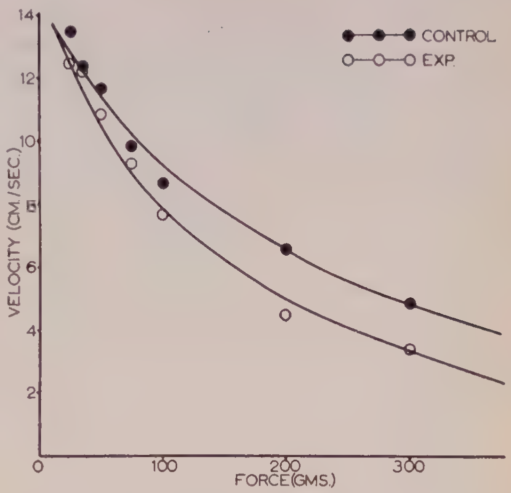

FIG. 1. Force-velocity curves of tetanus toxin shortened and contralateral control gastrocnemii. Circles are observed values; solid lines are theoretical curves.

Results. The solid lines in Fig. 1 represent the theoretical force-velocity curves calculated from Hill's characteristic equation, while the circles indicate actual observed values. Constants of the equation are presented in Table I.

In respect to maximum initial velocity, no statistically significant difference between toxin treated and control muscles can be demonstrated. Probabilities range from $P = .10$ to $P = .90$ for all observations. Although not within the accepted confidence limits, the probability $(< .02)$ of a difference between velocity constants *b* suggests a trend toward slower contraction in the experimental muscle. On the other hand, there is a clear cut, significant difference between the 2 'extra' heats of shortening as evidenced by the constants *a*.

Discussion. The present evidence that an economy in energy expenditure for shortening is effected by toxin treated muscles, makes it

TABLE I. Means and Standard Errors of Constants in Hill's Equation Derived for Tetanus Toxin Shortened and Contralateral Control Gastrocnemii.

	Control	Exp.	P
a (g wt)	246.5 ± 8.5	153.0 ± 11.0	.001
b (cm/sec.)	2.94 ± .18	2.50 ± .19	.02
a/P_o	.21	.17	

appropriate to reconsider the several aspects of altered mechanical properties previously noted in these preparations. To the extent that all such observations can be reconciled, one with another, some speculations concerning the fundamental basis for the changes seems desirable and warranted if meaningful future inquiries are to be initiated.

Tension deficit, either twitch or tetanus, is greater than expectations based upon reported atrophy(1), in this case 14%. Because of the considerably shorter equilibrium length (87% of control) in the experimental muscles, an approximation of the cross sectional area from the value of volume/length suggests little change in this index. Certainly, the negligible increase in water content recorded at 14 days post-injection cannot adequately account for the tension deficit. More pertinent, of course, are the depletions of myosin and potassium with concomitant increase of sodium reported by Fischer and Skowlund(8).

A greater decrement in twitch tension than tetanus tension (twitch tension 51%, tetanus tension 73% of control) produces an increased tetanus-twitch ratio(2). What are the characteristics of a toxin treated muscle twitch and tetanus response that may account for the difference in ability to maintain tension development? In experimental muscles the absolute twitch relaxation time relative to the control muscles is shorter. This observation, however, can be misinterpreted unless the actual twitch tension attained is considered. The twitch relaxation time is 73% of control while the peak twitch tension subject to decay amounts to only 51% of control. Thus, in effect, the average unit tension decay is prolonged. This phenomenon is not irreconcilable with the reported increase in maximum proportional rate of relaxation ($-dP/dt/P_o$), since the element $-dP/dt$ by definition describes maximum decay rate and this rate is not well maintained throughout the relaxation period. Indeed, a marked slowing of relaxation rate was noted before complete tension dissipation(2). The observation and interpretation are in accord with the findings of Ranson(9). Moreover, such a delay is evident, as well, in the contraction phase of the twitch response. Contraction time amounts to 90% of control for a total tension, as previously noted, equal to only 51% of control.

The critical fusion frequency—lowest stimulation frequency yielding nearly maximal tension together with complete fusion of the myogram — has been used to calculate the duration of maintenance of the active state at maximum intensity(10). In toxin treated muscles, the active state intensity parameter probably is diminished, but the evidence for a lower fusion frequency(2) implies that the duration parameter is prolonged.

Additional suggestive evidence for prolongation of the active state derives from the observation of an augmented semi-dynamic stiffness in the experimental muscles following bouts of tetanic stimulation. This augmentation is more noteworthy because in the unstimulated condition the semi-dynamic stiffness of these muscles is less than in the contralateral control muscles, *i.e.,* they exhibit an increased compliance. Ritchie and Wilkie (11) showed that the active state in frog sartorius muscles was prolonged by previous stimulation and that prolongation as well as tension development were more marked when the muscle was allowed to contract in tetanus against an increased compliance. From this evidence, one may speculate that in toxin treated muscles the twitch tension is the maximum attainable for the available active state intensity, whereas, the tetanus tension is, in fact, the supplemented response of the tetanus-twitch ratio and is attributable to active state prolongation.

Normally there is little reason to expect a decrease in shortening heat concomitant with active state prolongation. Indeed, Hill's studies(12) on the effects of anions (Br, NO_3, I) on muscle suggest the opposite effect with the distinct difference, however, that in these cases there is an increase in intensity parameter of the active state. When the intensity parameter decreases, as it appears to do in tetanus toxin treated muscles, shortening heat might logically be expected to decline. Each of the above responses can be interpreted in terms of depletion of myosin linkages acting as centers for energy release.

Summary. Investigation of the force-velocity relationship of muscles actively short-

ened by tetanus toxin injection reveals that these preparations exhibit a significant decline in the *a* constant of Hill's characteristic equation. An attempt has been made to interpret the above change in terms of other reported mechanical and chemical changes.

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Study of Lymph Lipids Following Administration of Oleic-1-C^{14} Acid with or without Cholesterol.* (24601)

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It has long been known(1) and confirmed (2-6) that when fatty acids are ingested either as free acids or in various compounds, they appear in lymph principally as triglycerides, the remainder consisting of small amounts of phospholipids, sterol esters and free fatty acids. In these various fractions specific activity has been found to vary according to fatty acid administered, but has generally been less than half that of the fed acid. It thus appears probable that incoming lipids are diluted in lymph in varying degree by endogenous lipids. Since in almost all cases in which labeled free fatty acids have been administered, they were dissolved in a large proportion of vegetable oil, it is probable that this difference between fed labeled and unlabeled acids has had an influence on distribution of activity in lymph lipids. In our experiments, rats were fed oleic-1-C^{14} acid entirely as free fatty acid, either alone or mixed with cholesterol, to assess the differences in lymph lipids brought about by presence of the latter substance during absorption of fatty acids. Lymph lipids were separated into various fractions by chromatography on silicic acid and, where possible, were hydrolyzed to give free fatty acids, which were separated on the reversed phase column of Howard and Martin. From weights and activities of all fractions, it was possible to determine, at least qualitatively, the influence of cholesterol on fatty acid absorption and distribution in lymph.

Materials and methods. Adult male rats weighing about 250 g were fasted for 12 hours before operation. The thoracic duct cannulation procedure was a modification of that described by Bollman, Cain and Grindlay(7). After operation, rats were given 1 ml of either oleic-1-C^{14} acid[‡] or a mixture of this acid with cholesterol (25 mol. %) by stomach tube.

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[‡] Fed oleic acid consisted of a mixture of oleic-1-C^{14} acid(8) and containing 6.3 x 10^4 disintegrations/sec/mg diluted with inactive oleic acid to mixtures containing 9.0 and 37.6 disintegrations/sec/mg. Composition of the acid determined by reversed-phase chromatography was 90% oleic, 10% linoleic.

TABLE I. Weights and Activities of Lymph Lipids following Administration of Oleic-1-C^{14} Acid. (269 mg total lipid chromatographed on 55 g silicic acid. Recovery: 224.7 mg. Activity of fed oleic acid: 9.0 disintegrations/sec/mg.)

Solvent, diethyl ether in pentane, %	Elution vol, ml	Components	Wt, mg	% of recovered lipid	Activity, dis/sec/mg	% of recovered activity
3	400	Sterol ester	7.1	3.2	.25	.1
8	300	Triglyceride	202.2	90.0	7.6	96.7
40	200	Diglyceride plus sterol	5.7	2.5	4.5	1.5
Methanol	50	Phospholipid	9.7	4.3	2.7	1.6

The rats were then placed in restraining cages with free access to 1% sodium chloride solution. Lymph was collected for 24 hours and lymph lipids were extracted by the method of Delsal(9). After evaporation of solvent, lipids were redissolved in pentane and separated by chromatography on silicic acid by modification of the method of Fillerup and Mead(10). Neutral lipids were eluted by pentane, mixtures of pentane with 3, 8, 25, and 40% ether and by ether. Phospholipids were removed from the column with methanol. Fatty acids obtained by hydrolysis of lipids by usual methods were separated on the reversed phase column of Howard and Martin (11,12). Counting of fractions was performed with Tracerlab C. E.-1 liquid scintillation counter operating at 55 ± 1.5% efficiency. The scintillation medium was 50 ml toluene (reagent) containing 4.029 g of 2,5-diphenyloxazole and 0.155 g of phenyloxazole reagent (scintillation grade; Arapahoe Chemical, Boulder, Colo.)/liter at 20°. All counts were corrected by comparison with a standard.

Results. After administration of 1 ml of oleic acid, about 500 mg of lipid could be recovered from lymph during the succeeding 24 hours (this appears to be a low recovery, but is within the range for various conditions and fats found by Blomstrand(13)). Chromatographic separation of this lipid on the silicic acid column revealed distribution shown in Table I. Specific activity of triglyceride fatty acids is 85% of that of fed acid, in contrast to results of Borgström(4), in which the labeled fatty acid was administered in olive oil and specific activity of lymph fatty acids was only 46% of that of fed acid. Fatty acids of the other lipids appear to suffer greater dilution with endogenous lipids.

In Table II are given results of a similar experiment involving administration of oleic acid-cholesterol mixture. In this case, as might be expected, the proportion of triglycerides was lower while those of cholesterol esters and phospholipids were higher, as re-

TABLE II. Weights and Activities of Lymph Lipids following Administration of Oleic-1-C^{14} Acid in Admixture with 25 Mol % of Cholesterol. (465 mg total lipid chromatographed on 55 g silicic acid. Recovery: 460 mg. Activity of fed mixture: 37.6 disintegrations/sec/mg.)

Solvent, diethyl ether in pentane, %	Elution vol, ml	Components	Wt, mg	% of recovered lipid	Activity, dis/sec/mg	% of recovered activity
0	50	Hydrocarbons	8.5	1.8	.24	.02
3	250	Sterol ester	47.5	10.3	22.0*	4.3
	125	Unknown	8.0	1.7	5.8	.5
8	200	Triglyceride	284.5	62.0	30.0	77.0
	125	Unknown	11.0	2.4	19.3	2.0
25	150	Cholesterol	9.0	2.0	17.4†	1.5
40	100	Diglyceride	20.0	4.3	30.0	5.8
100	100	Unknown (monoglyceride?)	11.0	2.4	19.7	2.1
Methanol	50	Phospholipid	60.0‡	13.0	12.2	7.0

* Activity of cholesterol ester fatty acids.

† This activity may be partially due to an impurity since it is unlikely that lymph cholesterol would be so active even after 24 hr.

‡ Only 13.6 mg of fatty acid was obtained by saponification.

TABLE III. Distribution of Fatty Acids of Lymph Lipids after Feeding a Mixture of Oleic-1-C^{14} Acid and Cholesterol.

	% fatty acid		
Lipid	Linoleic*	Oleic†	Stearic
Triglyceride	12.2	85.0	2.8
Cholesterol ester	20.4(14.0)‡	70.6(26.2)	9.0(8.7)
Phospholipid	36.8	50.0	13.2

* No attempt was made to separate palmitoleic or myristic acids from this peak. However, consideration of derivation of this lipid would indicate a small contribution from these acids.

† No attempt was made to separate oleic and palmitic acids, which occur together in this peak. However, consideration of derivation of this lipid would indicate a small contribution of palmitic acid, especially in triglycerides.

‡ Figures in parentheses are specific activities in disintegrations/sec/mg.

ported previously by Vahouny, Woo and Treadwell(14). The specific activities of both di- and triglycerides are about 80% of that of fed acids, but in this case triglyceride accounts for only 77% of total recovered activity in contrast to 97% when oleic acid was fed alone.

Distribution of fatty acids in various lymph lipids after ingestion of oleic acid-cholesterol mixtures is given in Table III. As might be expected from slight dilution of triglyceride fatty acids by inactive lipid, the distribution is very similar to that of fed acid. In the case of fatty acids derived from cholesterol esters of animals fed oleic acid-cholesterol mixtures, however, the greater dilution is reflected in a lower proportion of oleic acid and increased linoleic and stearic acids. The phospholipid fatty acids appear to have suffered even greater dilution, and proportions of linoleic and stearic acids are even greater. This observation appears at odds with the generally accepted idea that fatty acids of both these fractions are largely unsaturated. However, the relatively slight activity and low oleic acid content of the phospholipid fraction does not support a role of phospholipids in resynthesis of triglycerides during absorption and agrees with observations of Mead and Fillerup(15) that fed oleic acid is incorporated only to a small extent into blood phospholipids.

These results are consistent with the ideas that the presence of cholesterol during fatty acid absorption increases the proportions of endogenous lipids appearing in lymph. The endogenous fatty acids appear largely in the sterol ester and phospholipid fractions and consist, to a great extent, of oleic, linoleic and stearic acids. Presumably the endogenous phospholipid serves as a solubilizing agent for transport of the less polar sterol esters and triglycerides(16). The participation of stearic acid in such a process, however, remains obscure.

Summary. Oleic-1-C^{14} acid and a mixture of this acid with cholesterol were administered to rats with lymph cannulae. Chromatographic separation of lymph lipids in both cases revealed that the effect of cholesterol was to decrease the proportion of triglyceride and increase those of sterol ester and phospholipid. Chromatographic separation of fatty acids of lipids from the latter case revealed that although glyceride fatty acid distribution was similar to that of fed acids, both the sterol ester and phospholipid fatty acids were diluted with endogenous oleic, linoleic and stearic acids.

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Failure to Observe Alteration of Epinephrine Activity after Endotoxin. (24602)

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Zweifach, Nagler, and Thomas(1) have reported changes in epinephrine activity in blood vessels in the isolated rabbit ear and in mesoappendix of the rat following administration of endotoxin. In the rabbit ear they found that perfusion of 0.5 to 2 μg of endotoxin potentiated epinephrine reactivity and 40 to 50 μg caused reversal. Hinshaw, Gilbert, Kuida, and Visscher (to appear) were unable to demonstrate an altered vascular reactivity to epinephrine following injection of endotoxin in the isolated blood-perfused dog forelimb and lung. This prompted us to reinvestigate the responses in the isolated rabbit ear.

Method. The perfusion system was identical to that described(1), but in addition pressure recording equipment was used. The system consisted of a reservoir above a volumetric pipette calibrated in 0.1 cc. The ear was cannulated with a 22 gauge needle and connected to the pipette by a rubber tube. Tyrode solution was used as the perfusing fluid. Test doses in 0.1 cc were injected immediately upstream to the cannula. A needle in a polyethylene catheter was inserted into the rubber tubing at the level of cannulation, about 15 cm upstream, connected to a strain gauge, and the pressure recorded on a Sanborn twinviso recorder. The change in meniscus level was used to indicate alterations in resistance to flow through the ear. A rise in the meniscus level indicated increased resistance and increased perfusion pressure. Meniscus readings were taken at 1 minute intervals after epinephrine injections until perfusion pressure began to change. The meniscus was then readjusted to return the flow rate to the control value, *i.e.*, outflow from pipette was readjusted to constant inflow from the reservoir. It was required that the meniscus level remained stabilized for at least 1 minute before another injection of epinephrine. Lipopolysaccharide *E. coli* endotoxin from Difco Laboratories was used. The activity of this endotoxin was verified by assay in anesthetized dogs. Endotoxin and Tyrode perfusate were prepared with either laboratory distilled water for 3 experiments or in pyrogen-free water from Abbott Laboratories in the remaining experiments. No differences were noted.

Results. The epinephrine response after administration of a 0.5 μg endotoxin dose was studied 16 times in 12 ear preparations. Of these 12 preparations 4 ears were removed under ether anesthesia, 3 under nembutal, and 5 after decapitation. Flow rates varied from 2 to 3.4 cc/min. Absolute perfusion pressures were 37 to 92 cm H_2O in different preparations. Control epinephrine doses were 0.01 μg in 1 experiment; 0.001 in 10 experiments; 0.0001 in 3 experiments; and 0.00001 in 2 experiments. In several experiments control studies were performed to evaluate the consistency of epinephrine response (Table I). Usually only 2 injections were made with the same epinephrine dose prior to administration of 0.5 μg of endotoxin to decrease the length of the experiment to avoid excessive edema.

Zweifach *et al.* administered 0.5 μg of epine-

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TABLE I. Control Epinephrine Responses in Isolated Rabbit Ear.

Exp.	Change in meniscus level (mm) A. 0.01 μg	B. 0.001 μg
2-32	11.5	3.0
	10.0	4.0
	11.5	2.5
	10.0	1.5
2-33	7.5	.0
	7.0	.0
	5.0	.5
		.0
2-41	14.0	6.5
	9.0	3.0
	10.5	3.5
		5.0

phrine as a priming dose to test reactivity of the ear. In their sample protocol this dose elicited a 5.5 mm rise in the meniscus level. In our first preparation this epinephrine dose was injected and it gave such a marked response (171 mm rise in the meniscus level) that the pipette overflowed, the preparation never returned to control flow values and had to be discarded. In another ear a similar response was observed (178 mm rise, and an overflow of the pipette) and 42 minutes elapsed before the ear returned to control flow.

Only in 2 out of 4 experiments in which the ears were removed under Nembutal anesthesia, a small potentiation was observed after injection of 0.5 micrograms of *E. coli* endotoxin (Table II). Zweifach *et al.* reported observing 200 to 850-fold potentiation in the 5 experiments for which they presented data.

In 9 of the 12 ear preparations 50 μg of *E. coli* endotoxin were given either before or after investigating epinephrine activity with the small dose of endotoxin. Flow rates varied from 2.0 to 3.1 cc/min. In this series of 10 experiments the control dose of epinephrine was usually larger than those in our previous investigation using the smaller endotoxin dose. The response in 7 of the 10 experiments to the same dose of epinephrine tended to be less than the control response. A drop in the meniscus level was not seen, that is, a reversal of response was never observed.

The results obtained from the pressure recording of one preparation using the 2 different endotoxin doses are shown in Fig. 1. The change in perfusion pressure in millimeters of water is equivalent to the change in meniscus level. Flow rates were the same for both experiments. It is apparent that the epinephrine responses were essentially unchanged after either 0.5 or 50 μg of endotoxin.

Conclusions. 1) The fact that potentiation of the action of epinephrine after small doses

TABLE II. Epinephrine Activity in Isolated Rabbit Ear Associated with Administration of 0.5 μg of *E. coil* Endotoxin.

	Tests before endotoxin				Tests after endotoxin*							
Exp.	Dose	Response, mm H_2O	Dose	Response, mm H_2O	Dose	Response, mm H_2O	Dose	Response, mm H_2O	Dose	Response, mm H_2O	Flow, cc/min.	Perfusion pressure, cm H_2O
1 E	.001	1.5	.0001	.0	.001	1.5	.001	1.0	.001	1.5	2.7	57
2 E	”	3.0	”	1.5	”	1.0	”	1.0	”	3.0	3.1	47
3 E	”	3.0			”	1.0	”	2.5	”	1.0	3.1	48
4 E	.01	25.5	.001	7.0	”	5.5	”	7.5	”	7.5	3.0	68
5 E	”	30.0	”	2.0	”	1.5	”	2.5	”	2.0	2.4	47
1 D	.001	2.0	.0001	.5	.0001	.5	.0001	.0	.0001	.5	3.1	45
2 D	”	6.5	”	2.0	.00001	.0	.00001	.0			3.0	37
3 D	”	2.0	”	.5	.0001	.5	.0001	.5	”	.0	3.1	47
4 D	”	6.5			.001	6.5	.001	6.5	.001	7.0	3.0	37
5 D	.0001	8.0	.00001	3.0	.00001	3.0	.00001	3.0			2.7	39
6 D	”	8.0	”	2.5	”	3.0	.0001	8.5	.0001	7.5	3.0	50
7 D	.001	1.5	.001	1.0	.001	2.0	.001	1.0			2.9	34
1 N	.01	2.0	”	.0	.0001	.0	”	.5	.001	1.0	3.4	46
2 N	.001	1.0	.0001	.0	.001	1.5	.0001	.5	.0001	.0	3.3	51
3 N	”	2.0	”	.0	.0001	2.0	.001	2.0			2.0	92
4 N	”	1.0	”	.0	.001	1.5	.0001	.5	”	.5	3.1	43

* All doses were given within 14 min. after administering endotoxin.

Doses given in μg. E, ether; D, decaptitation; N, nembutal

TABLE III. Epinephrine Activity in Isolated Rabbit Ear Associated with Administration of 50 μg of *E. coli* Endotoxin.

Exp.	Tests before endotoxin		Tests after endotoxin*							
	Dose	Response, mm H_2O	Dose	Response, mm H_2O	Dose	Response, mm H_2O	Dose	Response, mm H_2O	Flow, cc/min.	Perfusion pressure, cm H_2O
1 E	.01	15.0	.01	7.0	.01	17.0			2.3	50
2 E	.001	2.5	.001	.0	.001	.5	.001	1.0	3.0	47
3 E	.01	124.5	.01	86.5	.01	94.0			3.0	45
4 E	.001	21.0	.001	19.0	.001	9.5	"	10.0	2.0	45
1 D	"	16.5	"	16.5	"	19.0			3.0	42
2 D	.01	109.0	.01	151.0					3.0	50
3 D	.0001	1.5	.0001	.5	.0001	.5	.0001	.0	3.1	37
1 N	.01	8.0	.01	8.0	.01	5.5	.01	12.0	3.1	50
2 N	"	7.5	"	5.0	"	6.0			2.0	92
3 N	.001	2.0	.001	2.0	.001	3.0	.001	3.5	2.8	40

* All doses were given within 13 min. after administering the endotoxin.
Doses are given in μg. E, ether; D, decaptitation; N, nembutal

of endotoxin was observed only to a slight extent in the presence of barbiturate, and not at all when ether or decapitation without anesthesia was employed to prepare the ear for perfusion, leads us to question whether the effect described by Zweifach, Nagler and Thomas is actually a direct potentiating action of epinephrine. We have no explanation as to how endotoxin might antagonize a barbiturate effect. 2) One point is noteworthy, our preparations in general had a much lower control epinephrine threshold than those reported for the perfused rabbit ear by above mentioned workers. 3) Our results are entirely uniform in showing that no reversal of epinephrine action after the large doses of endotoxin occurred, even with barbiturate anesthesia. We have no explanation to offer for this failure to confirm Zweifach *et al.*

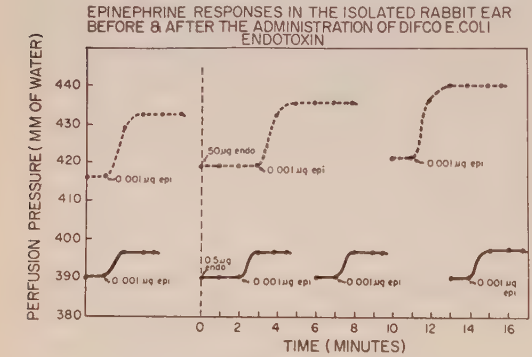

FIG. 1.

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Post Seizure Mortality Following Electroshock Convulsions in Certain Strains of Mice. (24603)

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Many phenomena associated with electrically-induced convulsive seizures in mice have been studied by investigators. Such factors as seizure threshold and experimental modifications thereof, seizure pattern, characteristics of postictal recovery period and effect of anticonvulsant substances on these various phases have been well described(1-5, and others). In spite of wide use of mice in such studies, little attention has been given to the fact that certain strains of mice are highly susceptible to a post seizure lethal effect of electroshock convulsions. Stone *et al.*(6) reported that among various strains of mice there was wide variation with respect to mortality following electroshock seizures. In that study a series of electroshock seizures was elicited in the same animal over several days. However, preliminary investigation showed us that multiple seizures were not required to induce death. In susceptible strains a single seizure may result in immediate fatal outcome. In view of this finding, as well as the fact that mortality in mice following electroshock seizures has not been well characterized, it was of some interest to study this phenomenon in more detail.

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Method. Electroshock convulsions were induced in mice of various strains by the method essentially similar to that of Swinyard *et al.* (1). In studying susceptibility of different strains to lethal effect of electroshock convulsions, the convulsive stimulus was delivered through saline-wick corneal electrodes. The stimulus, delivered from a Medcraft apparatus, type B2, was supramaximal in intensity (120v) and consisted of alternating current passed for 0.3 seconds. Care was taken to saturate the corneal electrodes with physiological saline at all times. When experiments studying mortality of various strains were conducted on different days, a control group of non-susceptible animals (CF#1) was studied concurrently. In the study of the relation between stimulus intensity and proportion of maximal seizures and mortality, different groups of mice were shocked with currents of varying intensities, such that the proportion of mice exhibiting maximal seizure increased from 0 to 100%. Square wave pulses of 2 msec. duration and frequency of 59/sec. were delivered through corneal electrodes for 0.3 second. The Offner electroshock apparatus, type 736-A, was used. The strains of mice included male and female CF#1 and CFW mice obtained from Carworth Farms, male and female mice of the ICR strain from the Inst. of Cancer Research and reared in these laboratories and male mice of the C57 and DBA strains obtained from Jackson Memorial Labs. Mice weighed between 14 and 26 g at time of experiment.

TABLE I. Post-Seizure Mortality among Various Strains of Mice Subjected to Maximal Electroshock Seizures.

Mouse strain		No. animals dying/No. tested*	% mortality
CFW	♀	31/40	78
”	♂	17/20	85
C57	♂	13/20	65
DBA	♂	18/20	90
ICR	♀	3/20	15
”	♂	4/20	20
CF #1	♀ †	0/70	0
”	♂	0/20	0

* All mice exhibited maximal seizure.

† Concurrent control animals, grouped for simplicity.

Results. Death in susceptible mice as a result of electroshock-induced convulsions occurred immediately after termination of maximal seizure. The apnea present during seizure appeared to be indefinitely prolonged. Autopsy performed when convulsion ceased, revealed that the heart was still beating in an apparently effective manner. Therefore, death may be ascribed to respiratory failure. Non-susceptible mice exhibited all characteristics of seizure pattern of susceptible mice, with the exception that respiration was promptly resumed at the end of the complete extensor phase of the convulsion. Recovery from that point was similar to that described for rats by Page(4) and more recently by Millichap(5).

A study of different strains of mice showed that there was wide variation in susceptibility to acute lethal effect of electroshock convulsions. The data in Table I indicate that DBA, C57 and CFW mice were considerably more susceptible than either the ICR strain or the CF#1 strain. The latter strain is used in this laboratory for routine anticonvulsant testing, and rarely has death followed electroshock-induced maximal seizures. The data in Table I also suggest that a sex difference did not exist with respect to susceptibility of death following electroshock seizures, at least in the particular strains tested.

The stimulus-response relationship between

TABLE II. Effect of Various Stimulus Intensities on Incidence of Maximal Seizure and Mortality in Female CF #1 and CFW Mice.

	CF #1 strain		CFW strain		
Avg current (m.a.)	No. of mice with M.S.* of 10 tested	No. of mice dying/No. with M.S.*	No. of mice with M.S.* /No. tested	No. of mice dying/No. with M.S.*	% mortality of those with M.S.*
4.2	0	0/0	2/40	1/2	50
5.0	3	0/3	10/40	6/10	60
6.0	7	0/7	21/30	13/21	62
7.2	9	1/9	20/20	18/20	90
8.4	10	0/10	20/20	13/20	62
25.1	10	1/10	20/20	17/20	85

* Maximal seizure.

intensity of stimulus and incidence of maximal seizure and mortality for a susceptible strain and non-susceptible strain is shown in Table II. As is to be expected, the data show that, as stimulus intensity was increased, number of mice exhibiting maximal seizure increased. Both the susceptible CFW strain and the non-susceptible CF#1 strain behaved in a quantitatively similar manner in this respect. The incidence of mortality following maximal seizure, however, was much more marked in the CFW strain, since only an occasional death occurred with the CF#1 strain.

The data in Table II also demonstrate that per cent mortality based upon number of mice exhibiting maximal seizure at different stimulus intensities, does not change over the range of intensities studied. Probit analysis showed that there was no significant regression relating per cent mortality of mice with maximal seizure with stimulus intensity ($X^2 = 2.68$, $P>0.1$, with one degree of freedom). Moreover, no deaths were recorded among mice which did not react with maximal seizure. The data indicate that the risk of dying following electroshock convulsions is related to maximal seizure and not to intensity of stimulus employed. Support for this concept is also given by the observation that the CF#1 strain survived current intensities as high as 400 m.a.(2).

Discussion. The phenomenon of post-seizure mortality in mice following electroshock convulsions has been mentioned(7-9), but has had little experimental attention. Mention is also made of the fact that certain drugs increased susceptibility of mice to post-seizure mortality. Reserpine(9), for example, increased post-seizure mortality, as has benactyzine(10). The present study demonstrated that post-seizure mortality varies considerably with the strain studied.

Swinyard *et al.*(1) have indicated that post-seizure deaths occur in a majority of mice in which electrode contact has been made improperly. This phenomenon, as observed by Swinyard and coworkers, resembles seizure fatalities reported here. In both instances, death resulted from failure of mice to resume breathing following termination of maximal seizure. Swinyard *et al.* speculated that, in the instance where improper electrode contact was made, severe respiratory depression resulted from current spread to the medulla. It is unlikely that a similar mechanism was involved in experiments reported here because: 1) Extreme care was used to maintain the saline-wick corneal electrodes fully saturated with saline at all times, 2) Concurrent control studies were always made with a resistant strain (CF#1), which rarely died, and 3) As shown above, post-seizure mortality was not directly related to intensity of stimulus, but occurred in constant proportion of those exhibiting a maximal seizure.

It is of interest that strains found most susceptible to post-seizure mortality after electroshock convulsions are identical with those susceptible to audiogenic seizures. The DBA and C57 mice have been widely studied in this latter respect. Moreover, both strains frequently die following audiogenic maximal seizure(11). The CFW strain has also been shown to be, at least in part, susceptible to audiogenic seizures, while both the CF#1 and the ICR strains are resistant (N.P. Plotnikoff, personal communication). Such findings suggest that susceptibility to post-seizure mortality following electroshock is genetically determined and may be related to factors which render certain strains susceptible to audiogenic seizures.

Summary. An acute lethal effect from electroshock convulsions has been demonstrated to occur among mice of the DBA, CFW and C57 strains, and to a lesser extent the ICR strains. CF#1 mice were rarely susceptible. Death was ascribed to failure to resume breathing and appeared to be dependent upon occurrence of maximal seizure and independent of intensity of current employed to elicit the seizure. The mechanisms involved were not revealed, but attention was directed to the finding that strains susceptible to post-seizure electroshock death are identical to those which are susceptible to audiogenic seizures.

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Gastrointestinal Absorption of Mg^{28} in Rabbits.* (24604)

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In a previous study a tracer dose of Mg^{28} was administered orally to human beings(1). Less than 10% of the radioactivity was recovered in the urine within 72 hours. Fecal excretion within 120 hours accounted for 59 to 88% of administered dose. The low renal excretion of magnesium was thought to be due to poor gastrointestinal absorption. The following study was undertaken to extend these observations in rabbits more clearly to delineate the metabolism of orally administered magnesium.

Material and methods. Domestic rabbits of mixed breeds were placed in individual metabolism cages and fed a stock diet of compressed pellets. Water was given without restrictions, except as indicated. About 200 μcuries of radioactive magnesium, Mg^{28}, contained in 30 to 35 meq of magnesium, was received weekly as acid solution of $MgCl_2$. This material was neutralized with 1N NaOH and the precipitate dissolved in 1N H_2SO_4. A slightly acid solution of $MgSO_4$ containing 5 meq of Mg in 150 ml of distilled water was fed to each rabbit. Radioactivity assays of tissues, urine and feces were made with a well scintillation counter and a scaler. External body surveys were performed with a directional scintillation counter attached to recording counting ratemeter. At least 10,000 counts were made on each sample. All determinations were corrected for physical decay. *Plan of experiments.* Each group of 6 rabbits was treated slightly differently, as follows: (a) In Exp. I, food and water were withheld for 17 hours. Water containing Mg^{28} was then left in feed pans for 24 hours. After

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* Supported by Contract between Univ. of Colorado and the U. S. Atomic Energy Comm., the Amer. Heart Assn. and Colorado Heart Assn.

Mg^{28} was supplied by Hot Laboratory, Brookhaven National Laboratory, Upton, L. I. N. Y., on allocation from U. S. Atomic Energy Comm.

[†] Established Investigator of Amer. Heart Assn.

TABLE I. Excretion of Mg^{28} in Rabbits Starved 36 Hours, Fed Mg^{28}, and Starved Thereafter.

	Urinary excretion (% ingested dose)					Fecal excretion (% ingested dose)		
				Cumulative				
Rabbit No.	0-24 hr	24-48 hr	48-72 hr	48 hr	72 hr	0-24 hr	24-48 hr	48-72 hr
7	5.08*					.0		
8	2.83	6.60†		9.43		.0	.0	
9	4.24*					3.5		
10	.78	4.82	2.96	5.60	8.56	.0	.0	.0
11	2.94	3.48†		6.42		.0	9.39	
12	6.97	7.06	2.48	14.03	16.51	.0	.0	.0
Mean	3.81	5.49	2.72	8.87	12.54	0.59	2.35	.0

* Killed for tissue analysis at 24 hr. † Killed for tissue analysis at 48 hr.

its removal no food or water were given thereafter. Feces and urine were collected at 24 and 48 hours and analyzed for radioactivity. (b) Exp. II. Six rabbits were starved for 36 hours. Water containing Mg^{28} was then left in feed pans for 24 hours and subsequently removed. The animals were starved thereafter. Urine and feces were collected at 24, 48 and 72 hours. External surveys of animals were performed at 24 and 48 hours. Two animals were killed by air embolism for tissue analysis at 24 and 48 hours. (c) Exp. III. Six animals were starved 48 hours; Mg^{28} was exhibited for 6 hours. The rabbits were then given food and water without restriction. External surveys were conducted at 24 and 48 hours. Pooled urine and feces were collected at 24, 48 and 72 hours.

Results. Exp. I. A mean of 1.3% of the ingested dose was excreted in urine in the first 24 hours; 8.8% during the second 24 hours. Fecal excretion during the first 24 hours was negligible; during the second 24 hour period it averaged 17.6%.

Exp. II. (Table I). The 24 hour urinary excretion of Mg^{28} averaged 3.8%. Only 1 of 6 rabbits had feces containing 3.5% of the ingested dose. External survey of the animals at 24 hours revealed maximal concentration of radioactivity over the midabdomen. Two animals (#7 and 9) were killed by air embolism and the entire gut removed; the remaining carcass contained minimal radioactivity. The caecum and its contents contained 78% of ingested dose; washed caecal tissue contained minimal radioactivity. 97% of ingested radioactivity was recovered in the carcass and the excreta of the one animal so studied (#7).

During 24 to 48 hours, urinary excretion of Mg^{28} in 4 rabbits averaged 5.5%; mean cumulative excretion for the first 48 hours was 8.9%. Only 1 of 4 rabbits had feces containing 9.4%. Maximal concentration of radioactivity was found on external survey over the midabdomen. Two animals (#8 and 11) were killed at 48 hours; most radioactivity was found in the contents of colon. The remaining 2 animals (#10 and 12) revealed an average urinary excretion during 48 to 72 hours of 2.7%. Mean cumulative 72 hour urinary excretion was 12.5%. No feces were excreted by either animal during this period. External survey at 72 hours revealed maximal concentration of radioactivity over the midabdomen.

Exp. III. (Table II). Mean urinary excretion during the first 24 hours was 7.3%; during the second period 3.9%, and from 48 to 72 hours, 1.8%. Mean cumulative renal excretions of radioactivity at 48 and 72 hours were 11.2 and 13.0% respectively. Mean fecal excretion of Mg^{28} at 24, 48 and 72 hours was respectively 46.5, 10.3 and 4.3%. External surveys over these animals at 24 and 48 hours revealed low concentrations of radioactivity over midabdomen, which were only slightly higher than those over the skull, *i.e.* the next highest concentration.

Comments. In most previous studies of gastrointestinal absorption of magnesium in man(2), large doses of Mg, on the order of 160 meq, have been administered; urinary recovery within 24 hours of 43% has been reported(2). Similar results have been reported in normal dogs, rabbits(2) and rats(3). In our study of magnesium metabolism in man (1) in which a more physiologic dose *i.e.* 3 to 10 meq of magnesium labeled with Mg^{28} was

TABLE II. Excretion of Mg^{28} in Rabbits Starved 48 Hours, Fed Mg^{28}, and Given Food and Water Thereafter.

Rabbit No.	Urinary excretion (% ingested dose) 0-24 hr	24-48 hr	48-72 hr	Cumulative 48 hr	Cumulative 72 hr	Fecal excretion (% ingested dose) 0-24 hr	24-48 hr	48-72 hr	Cumulative
13	4.95	3.68	3.27	8.63	11.90	21.05	11.24	11.85	44.14
14	10.18	4.07	2.54	14.25	16.79	43.77	12.85	6.66	63.28
15	6.07	2.46	.73	8.53	9.26	53.85	6.42	1.48	61.75
16	9.25	5.85	2.19	15.10	17.29	48.15	10.44	.74	59.33
17	5.15	2.41	1.03	7.56	8.59	49.12	5.62	1.48	56.22
18	8.04	4.91	1.14	12.95	14.09	63.15	15.26	3.70	82.11
Mean	7.27	3.90	1.82	11.17	12.99	46.52	10.31	4.32	61.14

administered as a single dose orally, a maximum of 6.3% of administered radioactivity was recovered in urine within 24 hours; these findings agreed with those of Pritchard(4). Our results in normal rabbits starved from 17 to 48 hours and given a small amount of magnesium orally are similar to those previously found in human subjects(1). The mean cumulative renal excretion of Mg^{28} during the first 48 hours after exhibition of the isotope in 3 experimental groups was similar, between 10 and 12.5% of ingested quantity, irrespective of length of starvation prior to exhibition of magnesium and regardless of whether food and water were given to animals after administration of Mg^{28}.

Fecal excretion of radioactivity was low when starvation was continued and maximum concentration of radioactivity was localized in the fecal content of caecum and colon. When food was given immediately after ingestion of Mg^{28}, fecal excretion of radioactivity increased markedly, whereas survey studies over the abdomen revealed an inverse decrease in concentration of radioactivity. The fact that duration of retention of Mg^{28} in the caecum and colon did not appreciably affect urinary excretion of radioactivity suggests that absorption of magnesium from the large gut was minimal and, indirectly, that most absorption of magnesium into the body had occurred *via* upper part of gastrointestinal tract.

Summary. Three groups of normal rabbits were starved for 17 to 48 hours prior to feeding of a dilute solution of $MgSO_4$. Mean urinary excretion of radioactivity in 48 hours in the 3 groups ranged between 10 and 12.5%. The high concentration of radioactivity detected in the midabdomen by external survey was traced to fecal content of caecum and colon. Fecal excretion was increased by resumption of feeding after exhibition of Mg^{28}, and concentration of radioactivity over the midabdomen decreased. These results suggest that poor gastrointestinal absorption of magnesium accounts for its low renal excretion. Absorption does not appear to occur from the large intestine.

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Sensitive Bioassay Method for Measuring Antidiuretic Hormone in Mammalian Plasma. (24605)

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Accurate measurement of antidiuretic hormone (ADH) titers in mammalian plasma has long been desired. Insensitivie assay methods and non-specific responses have plagued previous attempts to measure the circulating level of ADH under various physiologic conditions. In several existing methods, the assay animal has been a normal dog or a dog with experimentally produced diabetes insipidus(1,2,3). While these preparations are suitable for assay of relatively rich extracts of the neurohypophysis or hypothalamic nuclei, a more sensitive method is required for measurement of the circulating level of ADH in biologic fluids. Van Dyke(2) has stated that the rat assay is better suited for estimation of small quantities of ADH and that intravenous injections must replace the less specific intraperitoneal or subcutaneous routes. Assays of antidiuretic hormone in rats have been performed using both conscious and anesthetized animals. Ginsburg and Heller(4) used conscious rats in which a metal cannula was placed in the bladder, and a polyethylene tube in the jugular vein on day prior to assay. Urine collection consisted of prodding the animals into emptying their bladder every 5 or 10 minutes. It is extremely questionable if this procedure can be relied upon to produce complete emptying of bladder. Furthermore, animals were hydrated by stomach tube at frequent intervals. Considering the well known hypothalamic-neurohypophyseal interrelationships, the psychic influence of prodding and hydration would undoubtedly effect the endogenous release of ADH. For this reason, sensitivity of this method of bioassay must be questioned. Jeffers, Livezey and Austin(5), and Blackmore and Chester(6) rendered rats unconscious with ethanol. Their choice of anesthetic had the dual function of maintaining a steady state of anesthesia, as well as inhibiting endogenous release of ADH(7). A constant degree of hydration was maintained by administering fluid *via* a polyethylene tube passed into the stomach. This procedure presented a better physiologic approach to the problem of ADH bioassay. All previous methods(4,5,6) considered a decrease in urine flow as the criterion of antidiuresis. An important modification of this practice was suggested by Crawford and Pinkham(8) and by Thorn(9) in that urine density, rather than urinary flow rates, was used as index of response. This is especially important when spontaneous variations in urine flow are observed under control conditions. These variations may be due to either changes in glomerular filtration rate or solute load presented to the distal tubule. It is important to note that changes in urine specific gravity reflect only changes in absorption of water in the renal tubules. This alone could account for greater sensitivity in bioassay of ADH. It is also possible to measure specific gravity simply and with a high degree of accuracy. It was our purpose to combine the best features of existing methods of ADH assay, to verify previous data using standard solutions of vasopressin, and then to adapt the procedure further to measure circulating levels of antidiuretic hormone in mammalian plasma.

Methods. Holtzman strain female albino rats (220-280 g) were used as assay animals. On the afternoon prior to assay, the animals were given 3% of body weight of tap water at 40°C as suggested by Blackmore and Chester(5). All food was withheld during the night preceding the assay, but water was allowed *ad lib*. On day of assay, animals were prepared as described by Thorn(9). Following hydration with tap water and anesthetization with 12% ethanol (5% of body weight *via* stomach tube), the bladder was exposed with a suprapubic incision and cannulated by piercing upper portion with a 14 gauge hypodermic needle and threading a small piece of polyethylene tube down through the bladder and out the urethra. The bladder was then tied off above entrance of ureters to close the incision and reduce bladder dead space. The right exter-

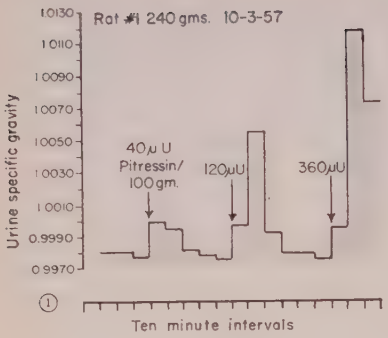
TABLE I. Antidiuretic Response to Doses of Reference Vasopressin.

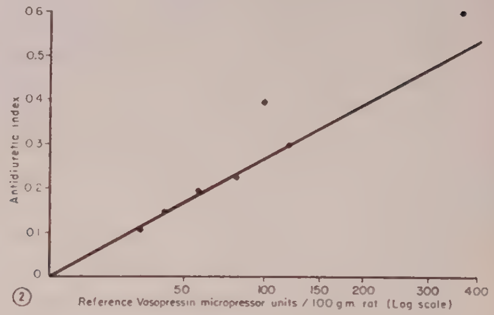
Dose of reference vasopressin, µpressor units/100 g rat	30	40	60	80	100	120	360
No. of observations	11	17	16	29	17	9	7
Mean antidiuretic index	.1088	.1454	.1910	.2213	.3901	.2964	.5945
Stand. dev. (±)	.0548	.0761	.0938	.0284	.0706	.0583	.0533

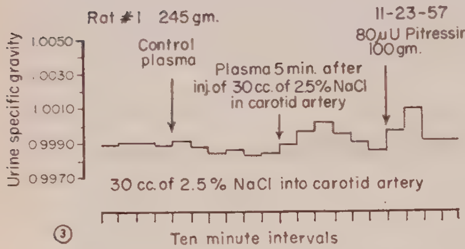
nal jugular vein was cannulated with fine polyethylene tubing for injection of all standard and unknown solutions. The stomach was cannulated *via* the esophagus for maintainance of constant degree of hydration and anesthesia with a 2% ethanol solution. At least 3 assay animals were prepared for every assay. It was sometimes necessary to discard an animal due to lack of sensitivity to standard solutions of vasopressin, or hematuria due to bladder surgery. Urine was collected in calibrated tubes under kerosene, and urine specific gravity was determined by the falling drop method(10). Degree of antidiuresis was expressed as antidiuretic index *i.e.* percentage change in urine specific gravity calculated from average of two 10 minute samples preceding injection, and the average of two 10 minute samples immediately following injection. One lot of Pitressin (Parke Davis & Co. R 983M, a lysine vasopressin prepared from combination of hog and beef pituitaries) served as reference material. Working standards were prepared fresh daily by dilution of the stock with physiologic saline. The volume of either standard or unknown solution injected for assay was fixed at 0.7 cc, the final amount regulated by dilution. After each injection, an additional 0.1 cc of heparin saline solution (1:100) was flushed through the tube to wash in all the test material. At least 60 minutes was allowed between injections to avoid any possible tachyphylaxis. Dogs were used as experimental animals. The neurohypophysis of the dog is drained by internal jugular veins; these veins contain blood richest in antidiuretic hormone. Due to extremely variable size of these veins, and the difficulty in sampling blood from the vein while maintaining a patent flow, blood samples were taken from catheter in the superior vena cava just above the right atrium. The catheter was placed using fluoroscopy. Ginsburg and Heller(4) reported blood from right heart of rat or from veins directly draining the posterior pituitary had equal levels of ADH activity. These authors reported that blood readily lost its detectable ADH activity upon standing, and thus should be rapidly injected for assay after withdrawal from animal. Blood to be assayed for ADH activity, was heparinized and immediately centrifuged after withdrawal from dog. 0.7 cc of dog plasma was slowly injected into external jugular vein of the rat and flushed in, as described above. In no case was time of blood withdrawal from dog to injection of plasma into the rat greater than 13 minutes. The usual time elapsed varied from 3 to 8 minutes. In all cases, the same rat was used for determination of ADH activity in control and experimental plasma samples. Thus, all variations in individual sensitivity were eliminated.

Results. Dose *vs.* response data for micropressor units of vasopressin *vs.* antidiuretic index is shown in Table I. A typical assay for standard doses of vasopressin is shown in Fig. 1. The immediate response to injection of standard vasopressin reflects the advantage of intravenous injections. The response is over in 30-40 minutes following injection, and thus a different standard or unknown can be tested every 60 minutes in the same rat. Antidiuretic indices obtained with this animal are included in the overall standard curve of log dose *vs.* response in Fig. 2. Linear results obtained by plotting antidiuretic index *vs.* log dose are clearly indicated.

Verney's classic experiment(11) was repeated wherein 30 cc of 2.5% NaCl solution was injected into the carotid artery of hydrated dog with average control urine flow of 1.28 ml/min. Five minutes later, the dog's plasma was assayed for level of circulating ADH when urine flow had fallen to a rate approximately 0.62 ml/min. Increase in specific gravity of rat's urine is much greater following injection of plasma after activation of the







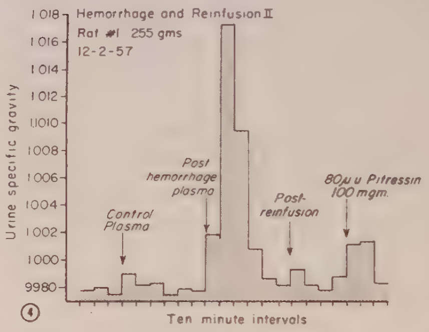


FIG. 1. Change in specific gravity of rat urine following intrav. inj. of reference vasopressin.

FIG. 2. Standard curve: Antidiuretic index *vs* log dose of reference vasopressin.

FIG. 3. Change in specific gravity of rat urine following intrav. inj. of dog plasma.

FIG. 4. Change in specific gravity of rat urine following intrav. inj. of dog plasma.

osmoreceptors, than the control plasma (Fig. 3). Thus, increase in circulating ADH occasioned by action of hypertonic salt solution on the osmoreceptors, resulting in reduction of urine flow of about 59%, is herein measured.

Sensitivity of this method of bioassay is indicated by series of experiments on hemorrhage. Following control determinations, the dog was hemorrhaged 25% of its estimated blood volume, then further blood samples were taken for determination of ADH activity. Following reinfusion of all heparinized withdrawn blood, plasma ADH levels were again determined. Results are given in Table II.

TABLE II. Antidiuretic Hormone (Microunit Equivalents Vasopressin/1.0 cc Plasma), 4 Experiments.

C_1	C_2	H_1	H_2	R_1	R_2
95	106	3764	750	29	15
22	14	3814	501	30	4
305	169	1350	3552		
33	73	1964	806	61	161

C = Control, H = Post hemorrhage, R = Post reinfusion.

When compared by analysis of variance, there is statistical significance to the 0.1% level between determinations taken during hemorrhage and those taken in control periods and following reinfusion. Fig. 4 is a typical assay experiment.

Discussion. Results obtained when comparing antidiuretic index to doses of reference vasopressin are in excellent agreement with Thorn(7). This is especially evident when different strains of animals and different lots of reference vasopressin were used. The report of Blackmore and Chester(5) of 45-105 microunit equivalents of vasopressin/cc of normal dog plasma is in close agreement with results obtained in this experiment. Again, differences in strains of animals and lots of vasopressin can account for much of the small difference.

Highly significant increase in circulating level of ADH following hemorrhage, had been reported by Ginsburg and Heller(9). However, these authors used unanesthetized animals for their assay, which was a highly unsatisfactory method for reasons mentioned above. Our method is far more sensitive and can be adapted to measurement of circulating ADH under various physiologic conditions.

Summary. A method of bioassay for small amounts of antidiuretic hormone in plasma of a dog is presented. The method utilizes rats rendered unconscious with ethanol, under constant water load, and with intravenous injections of all standards and unknowns. Change in specific gravity of rat urine expressed as antidiuretic index is used as criterion of potency. This method appears to be the most sensitive bioassay for ADH available in that small dose changes of reference vasopressin, or endogenous circulating ADH, can be differentiated.

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Hemosiderin. Iron Extraction Studies.*† (24606)

STEPHAN LUDEWIG (Introduced by Alfred Chanutin)
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Preparations of hemosiderin from horse spleens vary considerably in iron concentrations(1). Schwietzer(2) stated that part of hemosiderin iron may be present in crystalline form (limonite) as FeO(OH). After removing iron from hemosiderin with reducing agent, Greenberg(3) noted presence of apoferritin, thus indicating that this protein was originally present as ferritin. No other data are available concerning the form in which iron is present. This investigation is concerned with solubility of iron-containing compounds of hemosiderin in a chelating agent.

Methods. Hemosiderin was isolated from frozen horse spleens by fractional sedimentation as previously described(1). The procedure was modified by washing final product 4 times with distilled water. The washed hemosiderin pellet was changed to pasty consistency by addition of a few drops of water and the material stored in refrigerator in stoppered container. An aliquot of each sample was analyzed for total solids, iron and nitrogen. About 600 mg of "wet" hemosiderin were transferred to polyethylene bottle and 4 ml 9.2% disodium ethylenediamine tetraacetate (Na_2EDTA)/mg of iron were added. This mixture, to which 1 ml toluene was added, was continually agitated by magnetic stirrer. After centrifuging, 0.5 ml aliquots were removed, as a rule, after 1, 5, 15 and 25 days of stirring, for determination of iron. To release apoferritin from hemosiderin, we removed iron according to procedure of Greenberg(3). About 10 g of "wet" hemosiderin, equivalent to about 2 g of dried material, were suspended in dialyzing bag containing 60 ml of 1 M acetate buffer (pH 4.6), the bag then placed in 500 ml stoppered graduate filled with $Na_2S_2O_4$-acetate buffer and the cylinder rotated at slow speed. The reducing solution was changed every 12 hours. At end of 3 days the dialysate was practically free of iron. Subsequently, the material was dialyzed

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against cold tap water for 48 hours. To obtain a clear supernatant solution, the contents of bag were centrifuged at about 17,000 x g for 20 minutes; the residue washed twice with water and the washings combined with supernatant solution. Apoferritin was crystallized from the yellow-brown supernatant solution according to method of Granick and Michaelis (4). After adding cadmium sulfate, a brown amorphous precipitate which formed immediately, was removed by centrifuging at 17,000 x g for about one hour. A similar precipitate was not obtained when using a solution of apoferritin. The first crystals were observed after about 48 hours and continued to form on prolonged standing. Our routine procedure allowed this mixture to stand 6 days, centrifuge off the crystals, and allow the clear solution to stand for 3 more days. Additional crystals which formed, were removed and on further standing, crystals were no longer observed. Nitrogen content of collected crystals was determined. Iron was analyzed by wet ashing about 25 mg of dried hemosiderin in 2 ml concentrated H_2SO_4 and subsequent addition of several drops of 30% H_2O_2; 20 ml water were added and the mixture boiled 10 minutes to dissolve insoluble iron sulfate. This solution was brought to volume and aliquots analyzed for iron by adaptation of Laurell procedure(5). Iron extracted by Na_2EDTA could be determined without prior ashing provided excess phenanthroline was added. Nitrogen determinations were done by micro Kjeldahl procedure.

Results. Fig. 1 shows amount of iron extracted from "wet" and lyophilized dried hemosiderin suspended in 9.2% and 4.6% Na_2EDTA. An appreciable amount of iron, varying 24 to 28%, is extracted during first 24 hours; then iron is removed at a much slower rate. These results show that the most effective extraction of iron is attained with 9.2% Na_2EDTA- "wet" hemosiderin mixture. After 20 days 95% and 88% of total iron of "wet" and dry hemosiderin, respectively, is dissolved. Varying the ratios of volume of chelating agent/mg hemosiderin iron (2:1, 4:1, 8:1) did not affect amount of iron extracted.

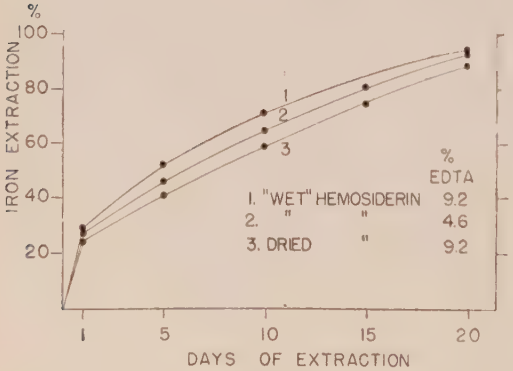

FIG. 1. Iron extraction from hemosiderin by Na_2-EDTA.

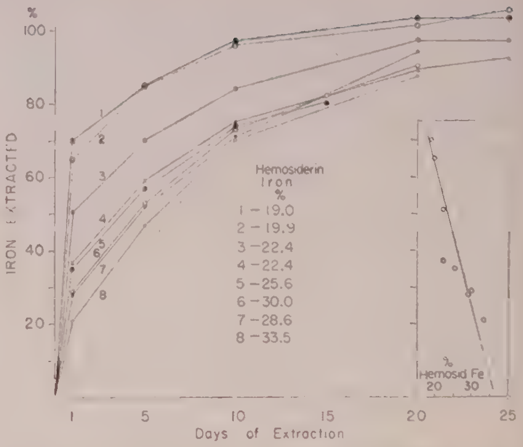

FIG. 2. Extraction of iron from 8 different hemosiderins by 9.2% Na_2EDTA. Data in insert show relationship between iron extracted during first 24 hr and total hemosiderin iron (19-33.5%).

Eight different preparations of "wet" hemosiderin from individual spleens were stirred in 9.2% Na_2EDTA solution for 25 days and per cent of extracted iron plotted against time (Fig. 2). There is an inverse relationship between amount of iron extracted at end of 24 hours and iron concentrations of hemosiderins. For example, 70 and 21% of hemosiderin iron is extracted from preparations containing minimum and maximum values of 19 and 33.5% iron. Parenthetically, it might be mentioned that iron concentrations for 9 different samples of hemosiderin were previously found to vary between 24.5 and 36.5%(1). The rate at which iron is dissolved after first day is related to initial hemosiderin iron concentration. The slow steady extraction continues until 90 to 100% of iron is removed from hemosiderin in 25 days. The results indicate that iron may be present in more than one form in hemosiderin. Iron that is readily dissolved during the first 24 hours undoubtedly represents a compound(s) which differs in composition from a relatively insoluble iron-containing residual material. To explain the gradual increase in iron after first day of extraction, it may be assumed that iron is firmly bound to an insoluble protein which undergoes structural changes during extraction or that an insoluble iron complex is gradually converted to a hydrate(6). The relatively insoluble iron residue is probably not identical with limonite(2) since this material is insoluble in Na_2EDTA.

In 6 samples of water-soluble fraction of iron-free hemosiderin, the apoferritin nitrogen concentration constituted 1.2 to 3.5% of total hemosiderin nitrogen. These results represent .5 to 1.5% of ferritin in hemosiderin. These results may not be quantitative since the most reliable data would be obtained by immunological technics. If it is assumed that all ferritin iron reacts with Na_2EDTA during the first 24 hours, it represents only a small portion of extractable iron.

Summary. 1. It has been demonstrated that iron is extracted from horse spleen hemosiderin at different rates by disodium ethylenediamine tetraacetate. During the first 24 hours of extraction, between 21 and 70% of total hemosiderin iron was removed by chelating agent from 8 hemosiderin preparations which contained between 33.5 and 19% iron, respectively. The amount of iron removed is inversely proportional to total hemosiderin iron concentration. Subsequently, iron is dissolved at a slow rate until practically all is removed during 25 days extraction. 2. Analytical data for amount of ferritin present in hemosiderin are presented.

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Strain Differences in Mercury Retention by Chicks.* (24607)

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Extensive information on pharmacology and toxicology of mercury is available(1,2). However, in a study of metabolism of organic mercury compounds, a difference in retention of mercury in strains of chicks was noted. This difference in strains of chickens selected for susceptibility and resistance to lymphomatosis is reported here. No reports have been found indicating a relationship between mercury retention and resistance or susceptibility to lymphomatosis. Pending further study, we do not imply such a relationship exists. No information is available on m.l.d. of phenylmercury acetate (PMA) with chicks. Löliger (3) fed a fungicide containing 5.1% phenylmercury pyrocatechol to hens at 1 g (26.5 mg mercury)/kg body weight without deaths. Hagen(4) reported the dose of same phenylmercury compound injected intravenously causing death to mice was 50-100 mg (25-50 mg mercury)/kg body weight. Basic phenylmercury nitrate injected intravenously at 8.6 mg mercury/kg body weight was fatal to rabbits(5). At Western Washington Exp. Station (WWES), intramuscular injections of 9 mg mercury at PMA/kg body weight caused

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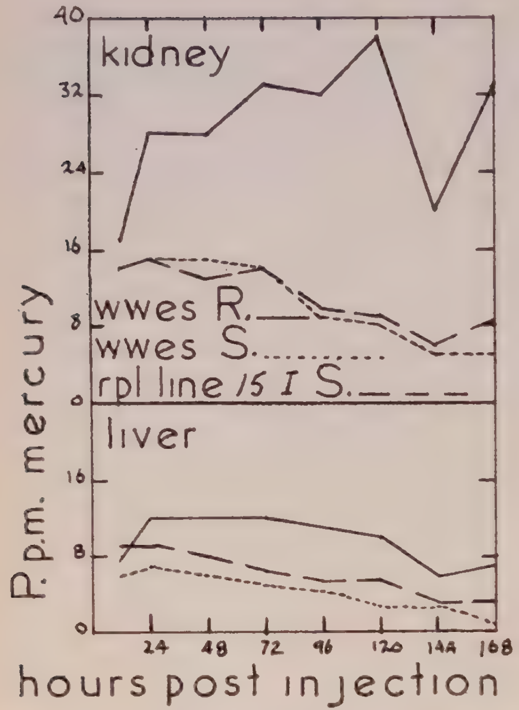

FIG. 1. Retention of mercury in livers and kidneys of resistant and susceptible birds following inj. of phenylmercury acetate.

no deaths in 20 chicks. Similarly per os, 18 mg mercury/kg caused 4 deaths in 23 chicks.

Methods. Chicks in Exp. 1 were 3 strains of White Leghorns. Two of these strains were selected from a common ancestry for resistance and susceptibility to visceral, neural, and ocular lymphomatosis incurred by contact exposure at WWES for 26 generations. Lymphomatosis mortality in the WWES Resistant (R) and Susceptible (S) lines resulting from contact exposure was very different for many generations, although recent generations have shown little difference. The third strain was Line 15 I of the U.S.D.A. Regional Poultry Research Lab. (RPL) East Lansing, Mich.,† a closed flock for many years, the offspring are highly susceptible to RPL-12 strain of lymphomatosis. In Exp. 2 only the WWES lines were used. *Exp. 1.* Male chicks were fed a practical type diet *ad lib.* from time of hatch. One hundred and thirty chicks, weighing 175 to 600 g, were injected intramuscularly in the breast with a solution of 3 mg/ml of recrystallized PMA in isotonic phosphate buffer at pH 7.2 at 3 mg mercury/kg of body weight. At intervals between 12 and 168 hours after injection, the chicks were decapitated and liver and kidneys excised and analyzed for total mercury(6).

Results. Results of mercury analyses are shown in Fig. 1. Concentration of mercury in liver tissue of the WWES R birds is greater than in the 2 S strains (WWES S and RPL 15 I). Analytical data for kidneys show an even greater difference between R strain and 2 S strains than do the data for livers.

Percentage of original dose found in liver plus kidneys for the same time was calculated. At 12 hours after injection, the average retention by all lines was 15 to 18% of original dose. With S lines, after temporary slight increases, the percentage retention decreased to 7% or less at 168 hours. R line retention increased to 34% at 72 hours, plateaued until about 120 hours, and declined to 23% at 168 hours post injection.

Analysis of variance of data from 96 birds that could be treated statistically, showed that concentration of mercury in livers was highly significant ($P < .01$) for time after injection and between strains. Similarly with data from kidneys, the differences for time after injection, between strains and interaction between time and strains were highly significant ($P < .01$).

Exp. 2 investigated retention of several types of compounds of mercury. The materials used were (1) PMA as in Exp. 1; (2) mercuric chloride in isotonic saline, with pH adjusted to 7.2 with isotonic sodium bicarbonate; (3) recrystallized ethylmercury chloride dissolved in isotonic saline; (4) sodium O-[(3 hydroxymercuric - 2 - methoxypropyl) - carbamyl] phenoxyacetate (Salyrgan theophylline Winthrop) diluted with sterile saline and standardized before injection. Salyrgan was selected because of reported very low retention of mercury by the animals(7). All mercurials were injected intramuscularly into the breast of WWES S and R birds at 3 mg mercury/kg body weight. Males weighing

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† The authors gratefully acknowledge cooperation of Dr. B. R. Burmester in furnishing hatching eggs of this strain.

TABLE I. Percentage of Injected Dose of Mercury Compounds Recovered in Liver and Kidneys of Chickens.

		% injected mercury recovered					
	Strain	24 hr		96 hr		168 hr	
Compound	bird	L*	K*	L	K	L	K
ØHgOAc	R	15.5	13.3	17.8	17.7	13.2	13.8
	S	9.8	8.7	4.8	5.2	4.6	4.7
$HgCl_2$	R	6.0	13.8	9.0	20.5	9.9	22.1
	S	4.9	6.7	3.8	6.7	4.3	5.8
EtHgCl	R	14.1	3.0	23.6	6.5	23.5	7.0
	S	11.1	2.8	19.5	4.1	17.8	4.1
Salyrgan	R	2.9	1.8	1.9	1.7	3.2	1.6
	S	1.0	.5	1.1	.8	2.9	.6

* L = Liver, K = Kidney.

166 to 285 g were used. The 92 birds involved were fed as in Exp. 1. After 24, 96, and 168 hours post injection, the birds were decapitated and liver and kidneys removed and analyzed for mercury.

The results in Table I indicate that chickens of the R strain retained more of the mercury compounds than did birds of the S strain. Mercuric chloride and PMA are retained in about the same 3:1 ratio by the 2 strains. With PMA the retention is about equal in liver and kidneys, whereas in birds injected with mercuric chloride, kidneys retained twice as much mercury as the livers. These data for liver and kidney retention with these 2 mercurials are in agreement with findings of Prickett *et al.* using rats(8). With ethylmercury chloride injections, smaller differences in retention were observed between livers of R and S birds. In contrast to preceding compounds, mercury was retained predominantly in the liver. Salyrgan mercury was retained only slightly by either line, athough, as with the other mercurials, the greater retention was in the R line.

From our data, phenylmercury and inorganic mercury appear to be metabolized in a similar manner. Phenylmercury appears to be broken down rapidly and thereafter is metabolized like inorganic mercury. Ethylmercury appears to remain intact longer in the animal body as suggested by Swensson (9). Salyrgan is excreted without decomposition to inorganic mercury(10). Our findings are in agreement with these reports.

While variation was observed in our experiments, except for post injection times of 24 hours or less, only on 2 individual observations was the lowest percentage retention in the R line less than the highest of either of the S lines in both experiments. Rate of absorption from injection site and establishment of equilibrium within the bird may account for short time variabilities. Strain differences in experimental animals may account for variability of other materials as well as mercurials.

Summary. Studies made with 3 strains of chicks showed one strain to be significantly different in retention of mercury in liver and kidneys, following intramuscular injections of phenylmercury acetate or mercuric chloride. Differences following injection of equivalent amounts of mercury as ethylmercury chloride or Salyrgan were much less. Mercury retention was greater in the strain selected for resistance to lymphomatosis than in the 2 susceptible strains which showed little difference.

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Morphine Antagonism with Amiphenazole (2,4-Diamino-5-Phenyl-Thiazole Hydrochloride) (24608)

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Shaw and Shulman(1) and others(2,3,4) report that concurrent administration of amiphenazole with morphine 1) permitted rapid increments in morphine dosage, 2) eliminated signs of development of tolerance or addiction to morphine, and 3) antagonized respiratory depressant effects of morphine. Shaw and Shulman(1) also point out that amiphenazole by itself is nontoxic in therapeutic doses and does not precipitate symptoms of abstinence from morphine in addicted patients. Fraser and co-workers(5) were unable to confirm these effects in man. This report presents data which confirm the report(6) that amiphenazole antagonizes morphine depression in dogs; the data also show that amiphenazole does not significantly affect morphine analgesia in mice.

Methods. Healthy dogs of both sexes were used. Morphine, 10 mg/kg, with hyoscine, 0.6 mg/kg, was given by subcutaeous injection. Usually analgesia sufficient to allow for minor surgical procedures and lasting several hours was obtained; unless severe morphine depression was obtained the animals were not used for testing. The compounds to be tested, amiphenazole and bromosulfolane, (3-bromotetrahydrothiophene-1, 1-dioxide)(7), were administered intravenously (i.v.). Dogs were observed for signs of arousal at least one hour and, if these appeared, they were watched for further period to determine whether depression recurred. The above procedure and the graded end points are those of Shaw and Bentley(6). In dogs anesthetized with pentobarbital sodium, mean blood pressures were recorded with mercury manometer. Amiphenazole was administered rapidly intravenously. *Analgetic activity* was determined in albino male Webster-Swiss mice by thermal method described by Chen and Beckman(8). Controls were run simultaneously each time that drugs were evaluated; all compounds were administered intraperitoneally (i.p.) to 10 mice at each dose level.

Results. In 4 anesthetized dogs, amiphenazole, 20 mg/kg caused average transient (less than 10 minutes) lowering of mean blood pressure of 47% (range 35 to 70). Atropinization (3 dogs) failed to alter this vasodepression (53%, range 37 to 75%).

Amiphenazole was more effective than bromosulfolane in counteracting morphine depression in dogs without inducing disturbing side effects. In 5 of 12 dogs the antagonism was outstanding: only 2 dogs failed to respond satisfactorily. Bromosulfolane was inactive in 4 dogs and showed good arousal in 2. Although amiphenazole is a central nervous system (CNS) stimulant, the dose of 20 mg/kg produced no signs other than respiratory stimulation in dogs depressed with morphine or in normal control dogs. This contrasts with results found in rats(9). On the other hand, bromosulfolane consistently produced bothersome CNS stimulant effects. Following amiphenazole about half of the dogs returned in ½ to 4 hours to a level of morphine depression somewhat less than that observed before drug. The results were not as consistent as those previously reported by Shaw and Bentley(6). In 15 dogs these investigators had good arousal in 12, mild arousal in 3, and no failures.

Amiphenazole, 100 mg/kg i.p. in mice, produced hyperexcitability, shaking, and ataxia following which the animals became quiet. At higher dosages, opisthotonos, threshold and clonic convulsions, loss of righting reflex, and salivation were observed. This was usually followed by moderate post-ictal depression, which is not uncommon for CNS stimulants.

Amiphenazole did not significantly alter the analgetic effects of morphine in mice (Fig. 1). Mice receiving combinations of morphine plus amiphenazole at 40 and 80 mg/kg were withdrawn and quiet, but not ataxic, at first 30

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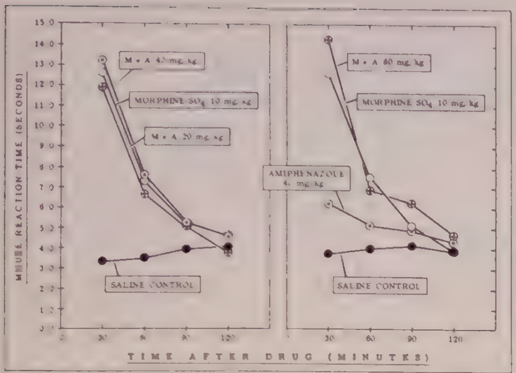

FIG. 1. Analgetic activity of morphine SO_4, amiphenazole and combinations of morphine SO_4, 10 mg/kg with various amounts of amiphenazole (M + A). All reaction times greater than 5.5 seconds were significantly greater ($P < .05$) than control. Each half of Figure represents independent experiment.

minute interval. This depression was not seen with morphine alone or with amiphenazole alone at 40 mg/kg. An unexpected and unexplained finding was that amiphenazole, 40 mg/kg, produced a significant ($P < .01$) analgetic effect itself at 30 minutes.

Summary. Amiphenazole was effective in antagonizing morphine depression in dogs; bromosulfolane was less effective even at toxic doses. Amiphenazole did not significantly alter the analgetic effect of morphine in mice, though this compound alone produced a mild but transient analgetic effect.

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Fibrinolysis II: Absence of Antibodies to Streptokinase Following Injection of Purified Human Plasmin in Rabbits.* (24609)

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Human plasmin (fibrinolysin) may be useful in control of thromboembolism. Thus it becomes important to establish its antigenic properties, since these could limit greatly its clinical use. This paper discusses the heteroantibody response in rabbits receiving purified human plasmin. Special reference is made to development of antibodies against streptokinase.

Materials and methods. (a) *Streptokinase,* a highly purified preparation, supplied by Dr. James Ruegsegger,‡ was stated to induce specific antigenic response when given intramuscularly to rabbits (Ablondi, personal communication). (b) *Preparation of human plasminogen (profibrinolysin).* Pooled citrated platelet-poor normal human plasma was stored at –20°C until used. After slow thawing in ice box, Fractions II and III of plasma were prepared by Cohn's method #6(1) (protein yield 1.8%), and Fraction III then obtained by

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* The authors wish to acknowledge help of Dr. Frank Ablondi, American Cyanamid Co., Pearl River, N. Y. and Dr. Daniel Kline, Yale Univ. School of Med. Some human plasma fractions (albumin and gamma globulin) were obtained through Am. Red Cross Blood Program.

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Cohn's method #9(1) (protein yield 2.7%). Plasminogen was prepared from Fraction III by the method of Kline(2). The final material was dissolved in distilled water (acidified with a few drops of N HCl) in the ratio of 1 mg of weight to 1 ml of water. (c) *Activation of plasminogen* was carried out as described by Kline(2). Streptokinase was added in the ratio of 2.5 units/mg of plasminogen. The plasmin preparation contained 88 mg of protein/100 mg of dry powder (Kjeldahl's method). Its lytic activity, as determined with lysin-methyl-ester technic (Ablondi, to be published) was of 19.7 U/mg protein. The activated plasmin was lyophilized and stored at 4°C. (d) *Technic of antigenic stimulation.* Lyophilized plasmin was dissolved in 2 ml of 0.0025 M HCl and then mixed with 2 ml of alumina gel. Five mg/kg weight were given intramuscularly to each of 4 New Zealand rabbits. Animals received a total of 6 injections at 3 day interval (average total 90 mg plasmin). All animals were bled from central artery of ear 10 days after last immunizing injection. Antigenic response was evaluated by complement fixation and agar plate precipitation technics. (e) *Complement fixation test* was performed by 100% hemolysis technic (3). Reagents used were Bacto complement (2 units in Meyer's gelatin buffer); sheep hemolysin (4 units in Meyer's gelatin buffer); animal's serum at serial dilutions in Meyer's buffer; and plasmin (mother solution of 10 mg/ml diluted to 1/5, 1/50, and 1/500 in Meyer's buffer). (f) *Agar plate precipitation test* was performed as described by Ouchterlony(4), using plates of 1.5% Bacto agar in 10% merthiolate in saline. Solutions containing 5 mg/ml were prepared in Sorensen buffer at pH 7.0 of human plasmin; human plasminogen; human albumin fraction; human gamma globulin fraction; and highly purified streptokinase. Human citrated platelet-poor plasma was also used at dilution of 1/20 in Sorensen buffer. Undiluted immune rabbit serum represented source of antibodies. All reagents were used in volume of 0.2 ml.

Results. Rabbits injected with purified human plasmin developed low complement fixation titers (1/32 or less) against the specific antigen. Results, however, were difficult to evaluate since plasmin is able to destroy complement activity. When the agar plate precipitation method was used, several identity bands developed against human plasmin, plasminogen and other components of plasma (Fig. 1). No reaction was evident against streptokinase, although we confirmed in rabbits the antigenicity of streptokinase injected in Freund's adjuvant.

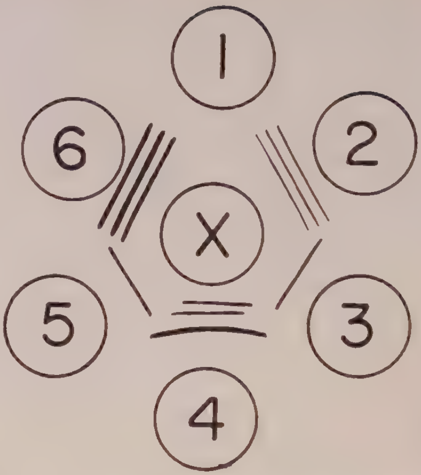

FIG. 1. Results of agar precipitation technic.*

* Central well (X): Serum of rabbit immunized against human plasmin. Peripheral wells: (1) purified streptokinase; (2) purified human plasmin; (3) human albumin; (4) purified human plasminogen; (5) human gamma globulin; (6) human plasma.

Conclusions. The results with the agar precipitation technic indicated the existence of multiple antigenic constituents of human plasmin, possibly due to contamination by other components of plasma. Our preparations of human plasmin, however, either contained no streptokinase or amounts of this agent insufficient to induce an antigenic response. Streptokinase represents a possible contaminant or constituent of plasmin. Thus, at least in our experimental condition, negative results are important as they eliminate one possible source of iso-antigenicity of human plasmin.

Summary. Agar precipitation technic detected no antibodies against purified streptokinase in rabbits immunized with streptokinase-activated, purified human plasmin.

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Effects of Surface-Active Agents on Carbohydrate Metabolism in Yeast.* (24610)

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Although inhibitory effects of surface-active agents on microbial metabolism are well known(1,2,3,4), several reports note that very small quantities of certain agents increase utilization of glucose, consumption of oxygen and production of CO_2(1,5,6,7). As far as is known, no detailed investigation of these stimulatory effects has been made.

Methods and materials. All experiments were performed on nonproliferating suspensions of commercial baker's yeast (Standard Brands) washed 4 times and aerated 4 hours prior to experiment. Gas exchange measurements were performed by standard Warburg technics. All suspensions were buffered with 0.03 M sodium succinate-tartrate adjusted to appropriate pH by addition of sodium hydroxide. With aid of commercially available glucose oxidase preparation (Glucostat—Worthington Biochemical Corp.) glucose utilization was determined by measuring disappearance of glucose from the medium. The highest concentration of benzalkonium chloride in yeast suspensions had no significant effect on glucose determinations. Most studies were made with surfactant, benzalkonium chloride, but in some experiments cetylpyridinium chloride, dodecylammonium chloride or sodium dioctyl sulfosuccinate were used. All surfactant concentrations are recorded as mg of surfactant salt/mg yeast. All Figures and Tables represent averages of 2 experiments.

Results. Effects of benzalkonium ion on anaerobic metabolism of glucose. The effects of cationic surfactant, benzalkonium chloride, on anaerobic CO_2 production from glucose at pH 5.5 are shown in Fig. 1. At benzalkonium ion concentration below 0.003 (mg benzalkonium chloride/mg yeast) anaerobic CO_2 production was only slightly inhibited. Inhibition became more marked with higher surfactant concentrations until at 0.006 surfactant concentration anaerobic CO_2 production was inhibited approximately 70%. For comparison with later experiments it should be noted that, in the range of 0.003 to 0.004 of surfactant concentration, inhibition increased from 14 to 35%. It is concluded (Fig. 1) that benzalkonium chloride was either without effect or was inhibitory to anaerobic metabolism of glucose in the range of surfactant concentrations used.

Effects of benzalkonium ion on aerobic metabolism. Effects on metabolism of glucose. In Fig. 2 are plotted data for aerobic CO_2 production and oxygen consumption as functions of benzalkonium chloride concentration. Both CO_2 production and oxygen consumption at first rose and then declined as surfactant concentration was increased. In the range 0.003-0.004 rate of CO_2 production rose about 1 μM/mg yeast/hr to peak value approximately twice the control value. Rate of oxygen consumption in 0.003-0.004 range showed a smaller rise of about 0.24 μM/mg yeast/hr, a 35% increase over the control. If it is assumed that CO_2 produced by process involv-

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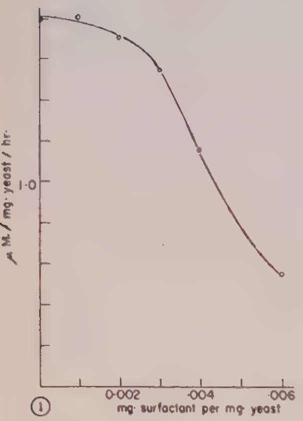
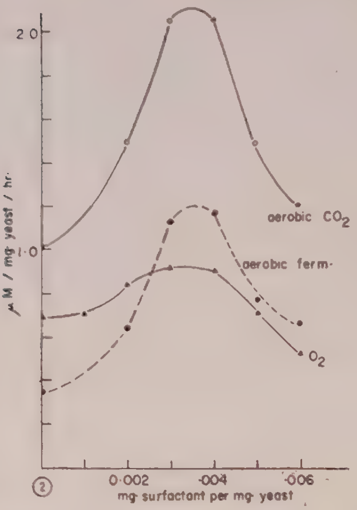
† Student Research Scholar of Kentucky Heart Assn.





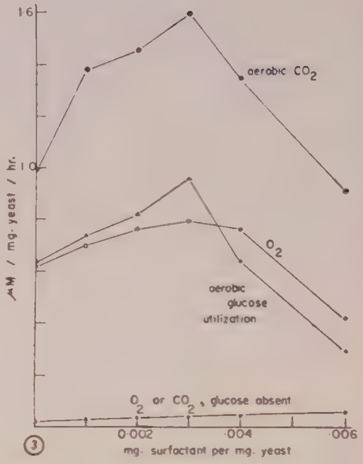


FIG. 1. Effect of benzalkonium chloride concentration on anaerobic CO_2 production by baker's yeast. Suspension contained 5 mg yeast/ml, 0.1 M glucose, 0.03 M sodium succinate-tartrate at pH 5.5, varying concentrations of benzalkonium chloride. Total volume was 3 ml; surfactant concentration expressed in terms of mg benzalkonium chloride/mg yeast. Warburg vessels gassed with helium prior to experiment.

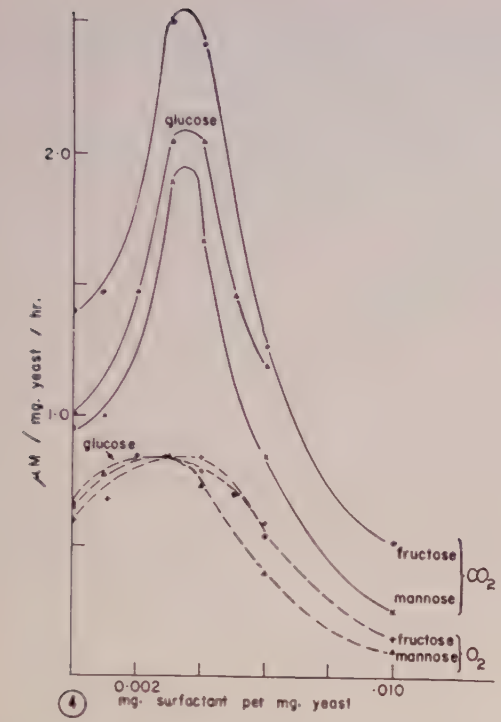
FIG. 2. Effect of benzalkonium chloride concentration on O_2 consumption and aerobic CO_2 production. Conditions same as for Fig. 1, except that air replaced helium in gaseous phase.

FIG. 3. Effect of benzalkonium chloride on oxygen consumption, aerobic CO_2 production and aerobic glucose utilization. Conditions same as in Fig. 2, except that initial glucose concentration was .03 M.

ing O_2 uptake (respiration) is in a one-to-one molar ratio with oxygen consumed, then the difference between rate of total aerobic CO_2 production and rate of oxygen consumption can be considered to be rate of aerobic fermentation, *i.e.*, rate at which anaerobic process (fermentation) is proceeding in presence of air. Thus, in the surfactant range 0.003-0.004 calculated aerobic fermentation rises to a maximum which is approximately 350% of control (Fig. 2). Increased aerobic CO_2 production in presence of benzalkonium ion therefore would be due primarily to an increase in, or a "release" of, fermentation by benzalkonium chloride.

Breakdown of carbohydrate stores with concomitant rise in CO_2 production and oxygen consumption was unlikely, since relatively negligible rates of CO_2 production and oxygen consumption (Fig. 3) were obtained at all surfactant concentrations when suspensions contained buffer but no substrate. Furthermore, comparison of rates of aerobic fermentation of Fig. 2 with rates of fermentation of Fig. 1 shows that aerobic fermentation never exceeded anaerobic fermentation at corresponding values of surfactant concentration; *i.e.* increased CO_2 production occurring in air when benzalkonium was added was limited by fermentation values found in anaerobic curve of Fig. 1.

Finally, a "release" of fermentation as the result of added surfactant would require that glucose utilization increase, to account for increased aerobic CO_2 production. Fig. 3 shows average results for 2 aerobic experiments in which rates of CO_2 production, O_2 consumption, and glucose utilization are plotted against surfactant concentration. It will be noted that rate of glucose utilization at peak value rose 0.3 μM/mg yeast/hr above control. The increase in rate of CO_2 production at peak value amounted to approximately 0.6 μM/mg yeast/hr above CO_2 control. Thus, for each extra mole of glucose utilized it appears that approximately 2 extra moles of CO_2 are produced, a ratio in agreement with stoichiometry of fermentation process. Since 6 extra moles of oxygen would need to be consumed for each extra mole of glucose utilized by respiration, it is evident that increase in oxygen consump-



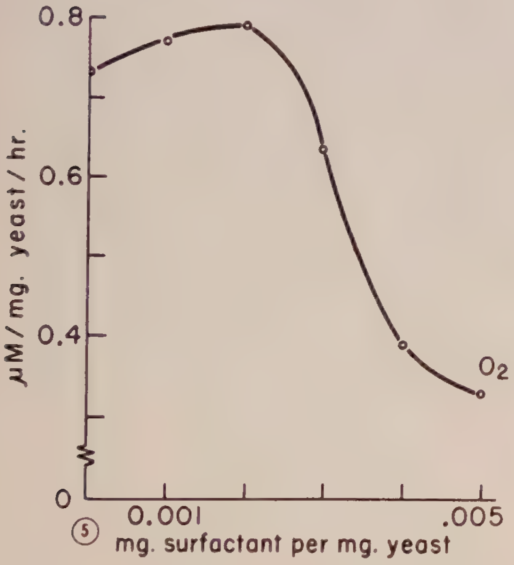


FIG. 4. Comparison of effects of benzalkonium chloride on aerobic metabolism of glucose, mannose, and fructose. Conditions same as in Fig. 2, except that in studies on mannose or fructose metabolism, appropriate hexose was substituted in equal concentration for glucose.

FIG. 5. Effect of benzalkonium chloride on oxidation of ethyl alcohol by baker's yeast. Conditions same as in Fig. 2, except that glucose was replaced by 0.02 M ethanol.

tion played a negligible role in increased utilization of glucose.

On the basis of experiments represented by Figs. 1, 2, and 3, therefore, it is concluded that appropriate concentrations of benzalkonium chloride bring about increased fermentation in presence of air.

Effects of benzalkonium chloride on the metabolism of other substrates. In Fig. 4 are shown comparative effects of benzalkonium ion on metabolism of glucose, mannose, and fructose. It is interesting that the same effect of surfactant was found with all 3 hexoses, both with regard to percentage stimulation of rates of CO_2 production and oxygen consumption, and with regard to surfactant concentration at which the peaks occur. In Fig. 5 results are shown for effects of benzalkonium chloride on oxidation of alcohol. In contrast to experiments with hexose substrates, oxygen consumption sharply declined in the surfactant range 0.002-0.004.

Comparison of effects of benzalkonium chloride and other surfactants on metabolism and effect of low pH. The effects of several surface-active agents on metabolism are summarized in Table I. At pH 5.5 all 3 cationic surfactants produced a maximum rate of aerobic CO_2 production. The peak occurred at different concentrations of surfactant in each case, however, and these differences in peak position do not correlate with molecular weights of the agents. At pH 5.5 the anionic surfactant, sodium dioctylsulfosuccinate, at 10 times the highest concentration of any cationics, produced no alteration of either rate of CO_2 production or rate of O_2 consumption. Cetyl pyridinium chloride at pH 5.5 and sodium dioctylsulfosuccinate at pH 2.5 inhibited oxygen consumption at all concentrations of surfactant tested (to 0.008 mg/mg yeast). This inhibition contrasted with effects of quaternary ammonium cationic agents which stimulated O_2 consumption.

At pH 2.5 benzalkonium chloride and sodium dioctylsulfosuccinate brought about a rise in CO_2 production, and in the case of benzalkonium chloride, a slight rise in O_2 consumption (see Table) was also noted.

When compared to curve of Fig. 2, the stimulatory effect of benzalkonium chloride at

TABLE I. Comparison of Effects of Surfactants on Aerobic Metabolism in Yeast. In addition to varying amounts of appropriate surfactant, suspensions contained 0.1 M glucose, 5 mg/ml yeast, 0.03 M sodium succinate-tartrate buffer.

Surfactant	Type	pH 5.5				pH 2.5			
		Relative surfactant conc. at peak stimulation and % stimulation at peak				Relative surfactant conc. at peak stimulation and % stimulation at peak			
		CO_2	% above control	O_2	% above control	CO_2	% above control	O_2	% above control
Benzalkonium chloride	Quat. ammonium cationic	.003–.004	105	.003–.004	31	.0005	35	.0005	10
Dodecylammonium chloride	*Idem*	.001	16	.001–.002	10				
Cetyl pyridinium chloride	Pyridinium cationic	.002–.003	60	No peak	Continuous decline				
Sodium dioctylsulfosuccinate	Anionic	*		——*——		.0005	41		Progressive inhibition

* No effect.

pH 2.5 occurred at lower surfactant concentration, but degree of stimulation at the peak was smaller than at pH 5.5.

Discussion. Figs. 1 and 2 indicate that 3 main factors in metabolism of hexoses are affected by benzalkonium ion: the controlling mechanism which is responsible for the Pasteur Effect, the fermentative process, and respiration.

Very low concentrations of benzalkonium ion inhibit the Pasteur Effect when the latter is measured either by glucose utilization or CO_2 production. Thus, there occurs an increased fermentation which is countered at higher benzalkonium concentrations by the inhibitory effect of the agent on the fermentation process itself. The presence of certain concentrations (0.003-0.004) of surfactant produces the expected peak in rate of CO_2 production.

The less pronounced but definite peak value reached in oxygen consumption when glucose was metabolized in presence of benzalkonium chloride is not explained. The fact that oxidation of alcohol (Fig. 5) was inhibited at surfactant concentration 0.003-0.004 makes it unlikely that increase in oxygen consumption is due to oxidation of the extra alcohol produced by "release" of fermentation. It is possible that benzalkonium ion produces stimulation in oxygen consumption by the fact that surface-active agents have a potent effect in disrupting the organization and permeability of the cell membrane(2,4,6). Increased permeability might then allow hexose substrate or an intermediate metabolite greater access to a reactive site. However, low concentrations of benzalkonium chloride were used in this study; whether these low concentrations have a great effect on membrane permeability has not been determined. In addition, increased oxygen consumption occurred only when the 2 quaternary-ammonium compounds were used, the other 2 compounds being strictly inhibitory to oxygen consumption. Perhaps, therefore, increased oxygen consumption is a specific effect of quaternary ammonium compounds rather than a nonspecific breakdown of a barrier to substrate penetration.

Since metabolism of mannose, glucose and fructose showed such similar responses to benzalkonium ion, the suggestion is made that there is a common "point of attack" by benzalkonium ion in metabolism of all 3 substrates. For example, benzalkonium ion might affect one or more enzymes in the pathway common to metabolism of all 3 substrates. Our findings are in agreement with those of Aisenberg and Potter(8) that the Pasteur Effect is mediated through inhibition of hexokinase or phosphohexokinase or both. Yeast hexokinase is known to phosphorylate all 3 hexoses(9,10). Since the release of fermentation by benzalkonium ion necessitates inhibition of the control factor or factors in the Pasteur Effect, the possibility arises that the normal "inhibition" of these enzymes is itself affected by the agent. Or again, the similarity of effect among various substrates could be a membrane or barrier phenomenon involving loss of structural integrity in the cell.

Within the range studied, cationic agents become increasingly potent as external pH is raised, wheras an opposite effect is seen for anionic type surfactants(1). As reported by us, the effects of the anionic agent, sodium dioctylsulfosuccinate, is in agreement with the literature. However, we found that the cationic agents tested produce their effects at lower surfactant concentrations when pH is lowered to 2.5. It may be conjectured that the pH tested is an extreme pH and that the cell is being subjected to abnormal stress at such low pH. It is well established that normal hexose metabolism by yeast is definitely inhibited at low pH(11). Although the effect of benzalkonium ion on metabolism occurs at lower surfactant concentration at pH 2.5 than at pH 5.5, the *extent* of the effect is much reduced at lower pH. The latter finding could suggest a directly inhibitory effect of hydrogen ion on metabolism of the injured cell.

Summary. 1. The effects of several surface-active agents on carbohydrate metabolism have been studied. Fermentation, oxygen consumption, and the Pasteur Effect are affected by these agents. 2. With the exception that oxygen consumption is affected differently by various surfactants tested, all 4 agents show qualitative similarities in their effects on carbohydrate metabolism. Benzalkonium chloride showed greatest effect on aerobic CO_2 production and the Pasteur Effect. 3. Several hexoses used as substrates showed the effect of benzalkonium ion to be non-specific in its action on metabolism of 3 hexose substrates. 4. Effects of the agents on oxygen consumption and effects of pH were discussed.

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Binding of an Antiviral Agent (Kethoxal) by Various Metabolites. (24611)

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β - Ethoxy - α - ketobutyraldehyde hydrate (Kethoxal) exhibits marked activity against several viruses in embryonated eggs(1,2). A logical sequence to demonstration of its antiviral activity in eggs was its evaluation in animal-virus systems. Kethoxal was tested against viruses of Newcastle disease in chickens, distemper in ferrets and influenza in mice. Although there was some indication of activity occasionally, it was of low level and inconsistent. The experiments reported here were designed to determine the reason for this lack of antiviral activity in animals.

Materials and methods. Kethoxal. Synthesis and some reactions of this compound have been reported(3). *Viruses.* Description of viruses used and egg technics involved in testing for antiviral activity have been described(2). *Microcolorimetric assay.* This was adapted from the procedure of Barrenscheen and Dreguss(4) for methylglyoxal. A standard curve was established by using weighed samples of Kethoxal. Kethoxal was precipitated as the 2,4-dinitrophenylosazone, which was washed successively with *N* hydrochloric acid and with increasing concentrations of ethanol in *N* HCl. The washed osazone was dissolved in 5% alcoholic potassium hydroxide and adjusted to volume. Model B Beckman spectrophotometer was balanced to zero optical density with alcoholic potassium hydroxide solution at wavelength of 565 mμ. The optical density of sample was then read at same wavelength. Average value obtained from triplicate samples represented one point on standard curve. The general procedure to assay for Kethoxal by this method in the presence of proteins, etc., can be illustrated by the reaction mixture of Kethoxal and normal chicken serum. One ml of aqueous solution of known Kethoxal content was mixed with 1 ml of normal chicken serum and 6 ml of 10% aqueous trichloroacetic acid solution was added. After centrifuging, the supernatant was transferred quantitatively to 10 ml volumetric flask. The protein precipitate was washed thoroughly with 2 ml of 10% trichloroacetic acid solution, recentrifuged, the supernatant added to the original and the whole made up to volume with 10% trichloroacetic acid solution. For analysis, a 5 ml aliquot was treated as described previously. Appropriate controls were made by diluting the serum with 1 ml of deionized water, deproteinizing, making up to volume as above and using 5 ml aliquot for analysis. The assay was accurate within ± 25% in the 5-30 μg range, within ± 10% in the 30-100 μg range and within ± 3% in the 100-200 μg range. *Alkaline peroxide assay.* This method was used for determination of Kethoxal in mg quantities. It was originally developed by Friedemann(5) and the application of the method to Kethoxal and its analogues has been described (3).

Results. Use of microcolorimetric assay to study Kethoxal binding. Since Kethoxal was active as an antiviral agent when injected into the allantois of chick embryos, a detailed study was made of its reaction with allantoic fluid, both *in vitro* and *in vivo*. Under conditions simulating those employed in routine administration of Kethoxal to infected embryonic eggs, Kethoxal at concentration of 0.5 mg/ml was added *in vitro* to normal allantoic fluid, harvested from 10 day eggs. For the *in vivo* study, a single dose of 2.5 mg of Kethoxal was injected into the allantoic cavity of the 10 day embryonic eggs and samples withdrawn at intervals and assayed for Kethoxal content. Rapid disappearance of Kethoxal from allantoic fluid *in vivo* is evident (Fig. 1). Other experiments(1,2) have indicated that there is marked antiviral activity when the same amount of Kethoxal is injected into eggs as much as 24 hours before the virus is injected. Thus, at time of infection there would be no detectable free Kethoxal present in the allantoic fluid, indicating that some bound form or some conversion product of

Kethoxal also showed antiviral activity. To learn more about the fate of Kethoxal when injected into various hosts, experiments were undertaken on recovery of Kethoxal added to body fluids and other substances *in vitro*. The general procedure in these recovery studies consisted of addition of a known quantity of aqueous solution of Kethoxal to a specified volume of serum, whole blood, etc. The mixture was deproteinized with aqueous solution of trichloroacetic acid, and an aliquot of the supernatant was analyzed essentially according to procedure described for chicken serum. It was found that Kethoxal was bound rapidly by blood, serum, urine, saliva, gelatin and other materials containing protein or protein-degradation products. Gelatin was used as a relatively simple model for more detailed studies of the reactions of Kethoxal with proteins. Two samples of sterile 10% gelatin, containing 1 mg of Kethoxal/ml, were prepared. One sample remained at room temperature throughout experiment; the other was kept at room temperature for 4 hours, then stored at –60°C. Aliquots of these samples were taken at intervals, assayed microcolorimetrically for Kethoxal content, and tested in eggs for antiviral activity. Similar experiments were also run using a synthetic gelatin hydrolysate consisting of a mixture of average quantities of amino acids reported to be present in gelatin. A 10% solution of this amino acid mixture was prepared, Kethoxal was added at a level of 1 mg/ml and disappearance of free Kethoxal and loss of antiviral activity were followed as described above. Results indicated (Table I) that Kethoxal was bound both by intact gelatin and by the synthetic gelatin hydrolysate, that rate of binding was approximately the same in the 2 cases, and that the bound Kethoxal was still active as an antiviral agent in eggs.

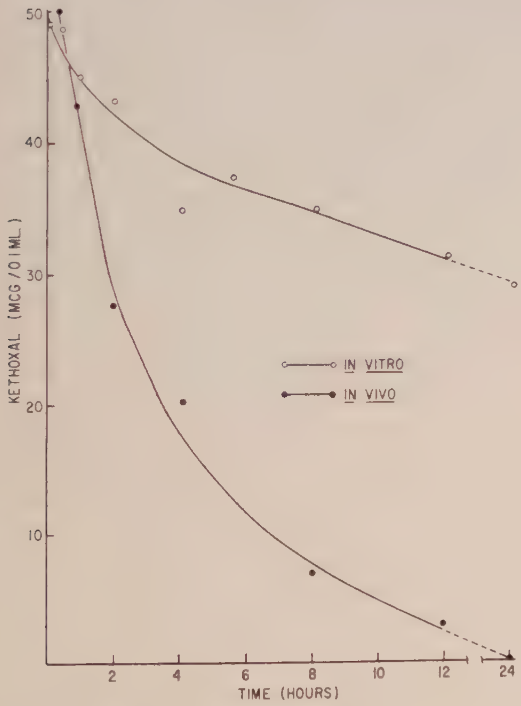

FIG. 1. Disappearance of Kethoxal from allantoic fluid.

TABLE I. Antiviral Activity of Kethoxal-Gelatin Mixtures.

Storage temp. (C)	Time after mixing (hr)	Free Kethoxal (µg/0.1 ml)	Egg activity† (% survivors)
	Mixture A*		
25°	0	100	94
	4	7	50
	8	0	10
	12	0	0
	24	0	6
25°/4 hr; –60°	8	0	47
	12	0	55
	24	0	35
	Mixture B*		
25°	0	100	100
	4	21	60
	8	1	12
	12	0	15
	24	0	6
25°/4 hr; –60°	8	20	50
	12	13	54
	24	16	36
Control		0	0

* Mixture A: 1 mg/ml of Kethoxal in 10% gelatin; Mixture B: 1 mg/ml of Kethoxal in 10% synthetic gelatin hydrolysate.

† Inj. 0.1 ml of test solution/egg into allantois of 10-day embryos; these eggs were infected 15 min. later with 25 lethal doses of Newcastle disease virus by the same route. Per cent survivors were recorded 10 days after infection. All tests for antiviral activity were carried out by our Virus Unit.

Reaction of Kethoxal with individual amino acids. Having established that Kethoxal reacted with the mixture of amino acids comprising the synthetic gelatin hydrolysate to yield biologically active products, it was of

TABLE II. Antiviral Activity of Kethoxal-Amino Acid Mixtures.

Amino acid*	Reaction time†	Free Kethoxal (μg/0.1 ml)	Egg activity‡ (% survivors)
Cysteine	5 min.	0	0
Arginine	5	0	5
Tryptophane	16 hr	28	0
Glycine	80	32	18
Lysine	63	23	16
Proline	140	32	76
Hydroxyproline	140	36	71
Histidine	20 min.	9	40
Glutamic acid	110 hr	18	79
Serine	25 min.	7	89
Threonine	25	7	100
Control		0	0

* Kethoxal (5 mg/ml) reacted with 10 molar equivalents of the amino acid at pH 7.4.

† Time required for Kethoxal concentration to drop to 2 mg/ml or less.

‡ Inj. 0.1 ml of the test solution/egg into allantois of 10-day embryos; these eggs were infected 15 min. later with 25 lethal doses of Newcastle disease virus by the same route. Per cent survivors were recorded 10 days after infection.

interest to examine the behavior of Kethoxal with individual amino acids present in this hydrolysate. An aqueous solution of Kethoxal at initial concentration of 5 mg/ml was treated with 10 molar equivalents of a given amino acid at pH 7.4 in 0.1 *M* phosphate buffer under sterile conditions. At intervals, aliquots of the reaction mixture were assayed for their Kethoxal content by the alkaline peroxide method. When the free Kethoxal content had fallen to less than 2 mg/ml, the remainder of the reaction mixture was diluted with sufficient sterile distilled water so that the quantity of free Kethoxal would be sub-effective in virus-infected eggs, but the total Kethoxal (free plus combined) would be sufficient to afford protection, providing the combined form retained antiviral activity. Such solutions, when assayed for antiviral activity in eggs, gave results which provided several points of interest (Table II). Kethoxal was inactivated very rapidly by cysteine and by arginine. With tryptophane the binding of Kethoxal took place more slowly, but biological inactivation occurred distinctly. Kethoxal bound to glycine and to lysine displayed antiviral activity initially, but when these samples were tested after 3 additional days at room temperature, they were inactive; this phenomenon is similar to that observed with Kethoxal bound to gelatin and to the synthetic gelatin hydrolysate. The rapid reaction of Kethoxal with serine and with threonine to yield products of marked antiviral activity was of particular interest. From the reaction mixture of Kethoxal with serine a crude solid was isolated. This material showed significant antiviral activity in eggs after being kept for a month at room temperature. Repeated attempts to obtain a homogeneous, crystalline product were unsuccessful. The antiviral activity of the Kethoxal-serine complex was inhibited completely by cysteine. Other hydroxyamino compounds, including serine methyl ester, homoserine and ethanolamine, also reacted with Kethoxal to give products showing high levels of antiviral activity in eggs; however, these, too, were inactive in animals.

Discussion. While gelatin-bound Kethoxal possessed antiviral activity in the embryonic egg system after free Kethoxal was no longer detectable (as was true of allantoic fluid-bound Kethoxal), the activity declined and finally disappeared after the reaction mixture stood at room temperature for a few additional hours (Table I). One interpretation of this behavior is that the initial reaction of Kethoxal with gelatin (or with allantoic fluid) effectively bound the ketoaldehyde with retention of antiviral activity; on continued standing, however, further reactions occurred yielding biologically inactive material. Such an interpretation is sustained by data shown in Table I when Kethoxal was allowed to react with gelatin at room temperature until Kethoxal disappeared (4 hours), and the reaction mixture was then held at –60°C during the next 20 hours. In this case, the antiviral activity of the conjugate was maintained for considerably longer time. This prolongation of biological activity is thought to be due to a retardation of the secondary reactions at low temperature. The rate of disappearance of Kethoxal in the synthetic gelatin hydrolysate closely simulated that observed for native gelatin; antiviral activity was likewise of the same order of magnitude. The Kethoxal-synthetic hydrolysate conjugate further paralleled the Kethoxal-gelatin conjugate in that bioactivity diminished after keeping the reaction mixture at room temperature for a relatively short time; however, antiviral activity could be prolonged by holding the reaction mixture at −60°C. Concerning the particular groups present in amino acids, peptides and proteins which are responsible for conjugation of these substances with Kethoxal, some concrete indications were obtained. Participation of primary amino groups seems clear from the observations that (a) Kethoxal reacted rapidly with simple primary amines; (b) Kethoxal reacted rather slowly with neutral α-amino acids, but with the corresponding α-amino acid esters rate of reaction was significantly increased; (c) in a given series of α-amino acids, rate of reaction with Kethoxal was retarded by loading the α-carbon atom with methyl groups; (d) no detectable reaction occurred between Kethoxal and N-acylated α-amino acids or N-acylated α-amino acid esters. The extremely rapid reaction of Kethoxal with arginine (and with guanidine) suggests the guanidino grouping as another site of reaction. Rapidity of reaction of Kethoxal with serine, threonine, cysteine and 2-aminobutanol-1, as compared to alanine, for instance, implies that a free primary amino group and an adjacent active group comprises another reactive area. In view of ability of Kethoxal to react rapidly with such a wide variety of chemical groupings, it was not surprising that it was rapidly bound and inactivated in the complex animal system. The fact that antiviral activity was retained in eggs was undoubtedly due to the much simpler chemical environment and low protein content of the allantoic fluid. It was thus concluded that the main reason for lack of antiviral activity of Kethoxal in animals was due to its participation in a variety of chemical reactions. To facilitate studies on fate of Kethoxal or its conversion products in animals, radioactive carbon-labeled Kethoxal was synthesized. A brief summary of results obtained with this material has been presented (1). A more detailed description, including methods of synthesis and its distribution, as well as excretion in mice, will be published.

Summary. Kethoxal (β-ethoxy-α-ketobutyraldehyde hydrate) reacted chemically with a variety of normal metabolites. Some of these reactions (*e.g.*, those with cysteine and arginine) resulted in rapid loss of antiviral activity; others (*e.g.*, with glycine and intact gelatin) in a more gradual loss of activity, while still others, particularly with serine and threonine, gave products possessing apparently undiminished activity. It was concluded that lack of significant antiviral activity by Kethoxal in animals could be attributed principally to its rapid binding and inactivation by proteins, amino acids and other metabolites.

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Cultivation of Enteroviruses in Hamster Kidney Tissue Culture. (24612)

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Enteroviruses(1) have recently been defined as consisting of polioviruses, Coxsackie and ECHO (enteric cytopathogenic human orphan) groups. ECHO viruses, with few exceptions, do not produce overt disease in monkeys, suckling mice, or other laboratory animals. To date, only tissue cultures of human or simian sources have proved of practical value in growth of enteroviruses. Interest in extending the range of infection of this group in tissue culture led to present investigation of the use of hamster kidney tissue culture. Hamster tissue was selected because of availability of the hamster, and the known susceptibility of suckling hamsters to Coxsackie infection. A cytopathogenic effect was obtained with certain enteroviruses and other viruses in this tissue culture system.

Materials and methods. Preparation of tissue cultures. Kidneys from adult golden Syrian hamsters were minced, trypsinized, and suspended in essentially the same manner as for preparation of monkey kidney tissue cultures(2). The cells were resuspended in growth medium consisting of 0.5% lactalbumin hydrolysate, 2% calf serum and 97.5% Hanks' balanced salt solution and 200 units penicillin, 200 μg streptomycin and 100 units mycostatin/ml. Prior to dispensing the cells, calf serum was added to make final concentration 8%. Following this method, the cells resuspended more evenly and had less tendency to aggregate than if they were resuspended in 8% calf serum immediately. Tube cultures were prepared with 1 ml of a suspension containing 500,000 cells. After 4 days' incubation at 36°C, the cultures were exchanged with above medium containing 4% calf serum.

At time of virus inoculation, the growth medium was replaced with 0.9 ml maintenance medium, consisting of 0.5% lactalbumin hydrolysate, 0.1% yeast extract (Difco), 0.2% bovine albumin (Fraction V, Armour & Co.), 2% calf serum, 97.2% Earle's saline, with antibiotics added as above. Cultures were inoculated with 0.1 ml undiluted stock virus. They were observed daily for 7 days. All viruses[†] were passaged 3 times in hamster kidney tissue culture before results were considered negative for virus cytopathogenic effect (CPE).

Results. Preparation of hamster kidney tissue cultures. Cultures were prepared using 4-8 kidneys. The yield of cells varied in different experiments, from 1.7-3.8 x 10^7 cells/kidney. The monolayer which developed after 6-7 days was not coalescent. Examination of stained preparations revealed the presence of 2 morphologically different cells (Fig. 1). Cultures consisted of spindle-shaped cells and small groups of large polygonal-shaped cells, which had a syncytial-like appearance. Nuclei of large polygonal-shaped cells were oval containing 1 to 10 elongated and irregularly shaped nucleoli. During 7 day observation period following inoculation, there was gradual degeneration of the culture, particularly after 4th-5th day. Increasing serum concen-

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[*] Markle Scholar in Medical Science.

[†] ECHO virus prototypes were obtained through courtesy of Committee on Enteroviruses(1). Poliovirus types I (Mahoney), II (MEF-1), III (Saukett) were obtained from Connaught Medical Research Labs, Toronto. Coxsackie prototypes A9, B1-B5 were obtained from N. Y. State Labs, Albany. ECHO 9 isolates through courtesy of Dr. C. E. van Rooyen, Halifax, (Linda S., Donald S.); Dr. O. Lahelle, Norway (V4162-7); Dr. A. J. Rhodes, Toronto, Canadian strains (309-56, 286-56), English strains (Squirrel, Haynes); Dr. R. Wigand, Cincinnati (Flack, Daniels). ECHO 9 (Hill) strain from Dr. W. McD. Hammon, Pittsburgh; Dr. S. S. Kalter, Montgomery; Dr. J. L. Melnick, Houston; Dr. H. A. Wenner, Kansas City. Other viruses studied were maintained in chick embryos in this laboratory: herpes simplex, HF strain; vaccinia, commercial calf-lymph vaccine; Newcastle disease virus-B strain; canine distemper virus—Onderstepoort strain; influenza A, Jap/305/57 and PR8/34.

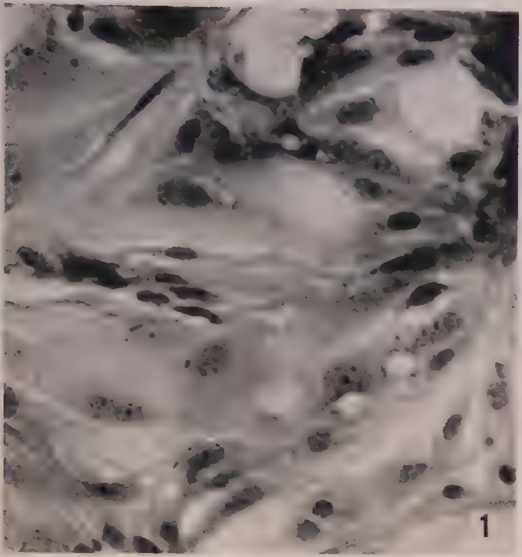

FIG. 1. Coverslip culture of hamster kidney, fixed in Zenker's solution and stained with hematoxylin-eosin. Large polygonal-shaped cells are surrounded by spindle-shaped cells. ×500.

tration to 3% aided maintenance of hamster cells. Eagle's medium in a base of Hanks' balanced salt solution or Earle's saline, plus 2%-3% calf serum also proved a satisfactory maintenance medium.

Susceptibility of hamster kidney cultures to enteroviruses. The results obtained with the enterovirus prototypes are summarized in Table I. None of the 3 poliovirus types produced any CPE. Of the 19 ECHO prototypes, a positive CPE was obtained for ECHO 10 and ECHO 9. Coxsackie viruses B1-B4 produced a rapid CPE (Fig. 2, 3), whereas B5 was slow in manifesting a cytopathogenic response. Strains other than prototypes of Coxsackie and ECHO viruses isolated in monkey kidney in this laboratory were inoculated into hamster cultures. Four Buffalo isolates of Coxsackie B2, 1 of B3, 8 of B4 and 13 of B5 were tested. All strains tested were positive. A 1957 Coxsackie B5 strain behaved like the prototype, producing a very slow degeneration of the cells. However, B5 strains isolated in 1958 in monkey kidney, produced a rapid CPE in hamster. The 2 A9 strains tested were negative, whereas the prototype had been adapted to hamster culture. Although the prototype ECHO 9 proved positive, most ECHO 9 strains were not cytopathogenic. Twenty-seven strains other than the prototype were tested, including 6 isolates from Norway, 2 from Halifax, Canada, 2 from Toronto, Canada, 2 from England, 2 from Ohio, and 13 isolates from Buffalo. Only 2 of these strains were positive. One was from Ohio (strain Daniels), and had been isolated contemporaneously with the prototype Hill strain (3). The other was isolated in Buffalo in 1957 from a household associate of a case of aseptic meningitis. In addition, prototype ECHO 9 (Hill) strain, obtained from 4 other laboratories and passaged once in this laboratory in monkey kidney, was cytopathogenic in hamster kidney tissue cultures. After 3 negative passages in hamster kidney, selected viruses were tested for 'silent growth', *i.e.* virus multiplication without manifestation of cytopathogenic effect, by inoculation of tissue culture fluid into monkey kidney tissue cultures. Negative results were obtained with ECHO 6 (D'Amori) and 10 non-cytopathogenic ECHO 9 strains. Polio virus type II was recoverable

TABLE I. Susceptibility of Hamster Kidney Tissue Cultures to Prototype Enteroviruses and Other Viruses.

Virus	Hamster passage 1	2	3
Enteroviruses			
Poliovirus types I, II, III	0	0	0
Coxsackie B1-B4	R	R	
B5	S	S	
A9	0	S	M
ECHO types 1-19	0	0	0
except: type 9	S	M	R
" 10	M	R	
Other viruses			
Herpes simplex	R		
Influenza PR8	0	0	0
Asian	S	M	R
Canine distemper	0	0	0
Vaccinia	R		
Newcastle disease virus	R	R	R

Viral cytopathogenic effect:

M—*moderate*, CPE not definite until 48-72 hr after inoculation; monolayer destroyed, but may take 96 hr after infection.

R—*rapid*, CPE definite at 24-48 hr after inoculation; monolayer destroyed within 48 hr after infection.

S—*slow*, CPE definite at 72 hr after inoculation; monolayer not destroyed 72 hr after infection.

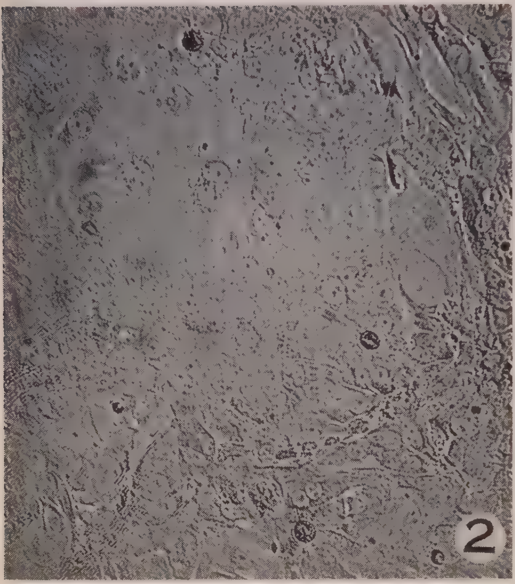

FIG. 2. Unstained T flask culture of hamster kidney. ×100.

in monkey kidney after 3 negative hamster kidney passages but not after 5. Polioviruses types I and III, after 3 hamster passages, were negative in monkey kidney. Negative results for canine distemper and influenza PR8 were confirmed by chick embryo infectivity tests.

Comparative sensitivity of hamster and monkey kidney tissue cultures. ECHO 9 (Hill), Coxsackie B2 and ECHO 10 stock monkey kidney viruses and hamster passaged viruses were titrated simultaneously in hamster and monkey kidney tissue cultures (Table II). ECHO 9 monkey kidney virus which originally titered 6 log TCD_{50} higher in monkey kidney became equally infective for both systems following 5 hamster kidney passages. Coxsackie B2 and ECHO 10 monkey kidney viruses were equally infective for both systems and this was unaltered after hamster kidney passage. Preliminary experiments, not presented here, have shown that monkey kidney and hamster kidney trypsinized cell suspensions can be mixed and grown together to form a monolayer. The large polygonal hamster cells were easily recognized in mixed cultures. Selective virus infection of monkey kidney cells in the mixed population has been obtained with poliovirus. Complete destruction of monkey kidney cells was noted, leaving hamster cells apparently unaffected.

TABLE II. Comparative Sensitivity of Hamster and Monkey Kidney Tissue Cultures.

	Tissue culture passage No.		Titer*	
Virus	Monkey	Hamster	Monkey	Hamster
ECHO 9 (Hill)	13	0	6.8	.8
	13	5	4.3	4.3
Coxsackie B2	6	0	5.8	4.8
	6	2	6.5	6.8
ECHO 10	16	0	3.5	4.5
	16	3	3.5	3.8

* Log TCD_{50}/.1 ml.

Susceptibility of hamster kidney cultures for isolation of viruses from clinical specimens. Isolation attempts were made simultaneously in hamster and monkey kidney tissue cultures, using clinical specimens from which Coxsackie viruses (B2, B3, B4, B5) had been isolated in monkey kidney tissue cultures during routine diagnostic studies in 1956-57. Stool suspensions, rectal and throat swabs and cerebrospinal fluid specimens were prepared as previously reported(4). Seven of 8 specimens tested were positive in monkey kidney whereas 4 were positive in hamster. During a study of aseptic meningitis in western New York in 1957, hamster kidney tissue

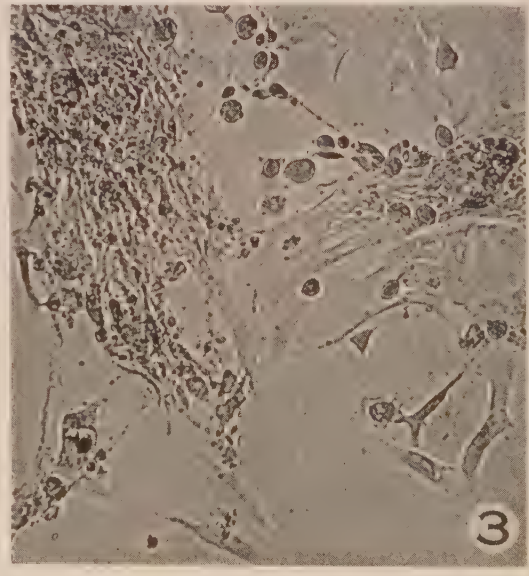

FIG. 3. Unstained T flask culture infected with Coxsackie B1 showing typical CPE. ×100.

cultures were used in diagnostic screening of monkey kidney isolates. Some isolates were cytopathogenic in hamster kidney and others were not, which suggested that the outbreak was due to a mixed group of enteroviruses, probably including Coxsackie viruses and ECHO viruses. Serologic tests later verified that hamster cytopathogenic isolates were, in all cases, Coxsackie viruses of group B, mainly type 4. Those isolates negative in hamster were identified as ECHO viruses, mainly ECHO type 9.

Discussion. Use of hamster kidney tissue culture in the study of several viruses has recently been described. Kissling(5) and Diercks and Hammon(6) have reported on growth of arthropod-borne viruses in this tissue culture system. The present report supplements the list of viruses cytopathogenic in hamster kidney tissue cultures, particularly in reference to members of the enterovirus group. Coxsackie viruses and ECHO viruses types 9 and 10 were cytopathogenic in hamster cultures. Polioviruses did not multiply, which is in agreement with results of Diercks and Hammon(6).

Wright(7) obtained a cytopathogenic effect in hamster kidney cultures inoculated with fluid of monkey kidney cultures containing measles virus isolated from a patient. Guerin and Guerin(8) studied susceptibility of adult porcine kidney tissue cultures to enteroviruses. The results are in agreement with those obtained for hamster kidney with the exceptions of Coxsackie A9 and ECHO 9, which were negative in porcine tissue. Warren and Cutchins(9) tested polioviruses and Coxsackie viruses in bovine embryonic tissue culture with negative results. The ECHO viruses were not examined in their study. Sheffield and Churcher(10) reported serial propagation of poliovirus in cells derived from rabbit embryo kidney. The interrelationships of members of enterovirus group to human and animal infection might be further explored with above tissue culture systems, since a gradient of sensitivity to infection is suggested from the results.

The results presented here and reported by others, show that hamster kidney tissue cultures can serve a very useful function in the diagnostic virology laboratory. This is demonstrated by the wide range of susceptibility of this system to virus infection and availability of tissue to most laboratories. In this study, recovery of Coxsackie virus in hamster kidney culture from clinical specimens was not as efficient as in monkey kidney.

Variation in susceptibility of hamster kidney tissue culture to infection with ECHO 9 isolates is difficult to explain. Other than the prototype Hill strain, only 2 strains of 27 examined were positive. Since approximately 10^6 monkey kidney TCD_{50} doses were required to initiate infection in hamster kidney with ECHO 9 (Hill) strain, susceptibility to infection seems to be of low order. This is supported by the fact that 10 ECHO 9 strains which failed to produce a cytopathogenic effect in hamster kidney did not grow 'silently' as shown by tests in monkey kidney. Certain ECHO 9 strains infect suckling mice, causing myositis typical of Group A Coxsackie infection, and it has been reported that large doses of virus are necessary to initiate infection in this host(11). The adaptability of a few ECHO 9 isolates to hamster kidney tissue culture further suggests the possibility of relationship of ECHO 9 to the Coxsackie group. The positive results with ECHO 10 supplement the evidence that this virus is biologically distinct from other members of the ECHO group. ECHO 10 virus has been shown to infect suckling mice and chick embryos and to be larger in size than other ECHO viruses (12,13). In addition this virus produces a characteristic pathologic change in monkey kidney tissue culture which differentiates it from the other enteroviruses(14).

Summary. Susceptibility of hamster kidney tissue culture to enteroviruses was studied. A cytopathogenic effect was obtained with the prototype Coxsackie viruses (A9, B1-B5) and prototype ECHO virus types 9 and 10. Variability of infectivity was shown for ECHO 9 isolates, only 2 of 27 positive. Other ECHO serotypes and polioviruses were negative. Strains of herpes simplex, vaccinia, Newcastle disease virus and influenza (Asian), also produced a cytopathogenic effect in hamster kid-

neys. Use of hamster kidney tissue cultures as a selective system in identification of agents from cases of aseptic meningitis was reported.

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Incidence of Liver Tumors in Male and Female Rats Fed Carcinogenic Azo Dyes. (24613)

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Sex and sex hormones have an influence on carcinogenesis in the liver. Female mice develop more liver tumors than males following administration of *O*-aminoazotoluene(1). Testosterone administration lowers this incidence (2), and stilbesterol-treated female mice develop liver tumors more readily than untreated females when fed diets containing *O*-aminoazotoluene(3). However, male rats of several strains are more susceptible to development of liver tumors than females, when they are fed diets containing acetylaminofluorene (4,5,6). Working with carcinogenic azo dyes, Rumsfeld *et al.*(7) find a higher incidence of liver tumors in male than in female rats fed 3′-methyl-4-dimethylaminoazobenzene or 4′-fluoro-4-dimethylaminoazobenzene. The present report describes the relative incidence of liver tumors in male and female Osborne-Mendel rats fed azo dyes, *p*-dimethylaminobenzene-1-azo-1-naphthalene (DAN), *p*-dimethylaminobenzene-1-azo-2-naphthalene (DA-2-N), or *p*-dimethylaminoazobenzene (DAB).

Materials and methods. Male and female Osborne-Mendel rats, 3-4 months old, were fed a semisynthetic diet containing *p*-dimethylaminobenzene - 1 - azo - 1 - naphthalene (DAN), *p* - dimethylaminobenzene - 1-azo-2-naphthalene (DA-2-N), or *p*-dimethylaminoazobenzene (DAB). The diet consisted of cerulose sugar 78%, vitamin-low casein 12%, salt mixture 4%, corn oil 5%, and vitamin mixture 1%. The composition of the vitamin mixture in mg % was thiamine hydrochloride 30, riboflavin 20, choline chloride 300, calcium pantothenate 70, pyridoxine hydrochloride 25, and nicotinic acid 50. In addition each animal received one drop of halibut liver oil once a week. Treatment of rats in each experiment is tabulated in Table I. *DAN*: Seventy-five male and 60 female rats received 0.075% DAN in the diet for 300 days. Ten rats each received respectively, 0.15, 0.3, and 0.6% DAN for 300 days. *DA-2-N*: Forty male and 24 female rats were fed a diet containing 0.075% DA-2-N for 230 days. *DAB*: Eighty male and 40 female rats were fed diets containing 0.06% DAB. Another group of

TABLE I. Incidence of Liver Tumors in Osborne-Mendel Rats Fed Carcinogenic Dyes.

Treatment				Tumors induced			
Carcinogen			Induction	♂		♀	
	%	Days fed	period (days)	Incidence	%	Incidence	%
DAN	.075	300	270	43/57	75	8/48	17
	.15	”	”			1/9	11
	.3	”	”			3/5	60
	.6	”	”	2/4	50		
DA-2-N	.075	230	80	39/40	98	24/24	100
DAB	.06	280	150	56/66	85	6/31	19
DAB and protein	.06 8	”	”			2/52	4

60 female rats receiving 0.06% DAB were given 8% protein in place of the usual 12% in their diet. All animals were placed on a diet of purina chow pellets, when their carcinogenic diet was discontinued. Daily observations were made on diet consumption and general health of each rat. Animals were palpated for tumor growth and weighed every 2 weeks, and killed for study when tumors became large, or when rats appeared very sick. All clinical diagnoses of tumor were confirmed by histologic diagnosis after postmortem examination. Multiple sections were examined from all livers and from all conspicuous tumors. Tissues were fixed in Zenker-acetic and microscopic sections were stained routinely with hematoxylin and eosin.

Results. Number and percentage of liver tumors induced in each experiment with azo compounds DAN, DA-2-N, and DAB are shown in Table I. DAN and DAB induced more liver tumors in male than in female Osborne-Mendel rats; while DA-2-N fed rats showed no significant sex difference in incidence of these tumors.

The first liver tumor was palpated on 270th day in DAN fed rats, on 150th day in DAB fed, and on 80th day in DA-2-N fed rats, after start of the carcinogenic diet. "Induction period" is defined as the interval between beginning of experiment and appearance of first palpable tumor. All incidence figures are based on number of animals that survived the induction period.

The 3 carcinogens used, differ from one another in having a benzene, an *a*-naphthalene, or a *b*-naphthalene radical, and are similar in having *p*-dimethylaminobenzene with an azo linkage. When an equimolal weight of each carcinogen was fed to different groups of rats, no significant difference was observed in diet consumed, general health, or weight during the induction period.

Differences in induction periods of these compounds probably reflect the differences in metabolism, detoxification, and elimination of the compounds and capacity of liver to carry on these functions. There have been a number of reports about metabolism of DAB by the rat liver, but no such information is yet available for DAN and DA-2-N.

In DAN fed rats, 57 males survived the induction period and of these 43 developed liver tumors, an incidence of 75%, while only 8 out of 48 female rats had liver tumors, an incidence of 17%. The average time for appearance of palpable tumors in males and females was 445 and 538 days respectively. Microscopic examination of liver tissue showed no significant difference in incidence or extent of cirrhosis, fatty changes, bile duct cysts or cholangiofibrosis, between livers of male and female rats. However, as female rats came to necropsy about 3 to 4 months later than male rats, their lesions have taken that much longer time to develop.

Female rats receiving twice as much DAN (0.15%) as in the first experiment, did not show any increase in incidence of liver tumors. However, when the dose of carcinogen was further increased to 0.3 and 0.6%, only about half of the animals survived the induction period, and none survived over a year. In rats fed 0.3 and 0.6% DAN, about half of survivors developed liver tumors. Increasing the dose of DAN failed to shorten the induction

TABLE II. Time of Appearance of Palpable Liver Tumors in Rats Fed DA-2-N.

Time in days	Tumors palpated			
	♂		♀	
	No.	%	No.	%
Under 210	4	10	3	12
211-270	27	69	4	17
271-330	6	15	10	42
>331	2	5	7	29

period of 270 days for this compound. Daily food consumption by rats fed higher doses of DAN was not significantly different from that of rats on 0.075% DAN in the diet. Since effective higher doses of the carcinogen failed to shorten the induction period, it appears that concentration of DAN was not the limiting factor in induction of tumors in these experiments. Rates of metabolism of the azo dye, detoxification and elimination of metabolites, and depletion of reserve capacity of liver to perform these functions are some factors which may determine the pace of tumor induction.

In DA-2-N fed rats 39 out of 40 males and all 24 females developed liver tumors, an incidence of 98 and 100% respectively. Tabulation of time of first appearance of palpable tumors (Table II) reveals that in 9 months 79% of male rats had palpable tumors while only 29% of females developed palpable tumors.

Relative delay in appearance of tumors in female rats indicates a higher resistance to carcinogenesis induced by DA-2-N. If the experiment had been terminated at 270 days, it may have reflected some of this resistance in incidence figures.

In rats fed DAB, 56 out of 66 male rats and 6 out of 31 females developed liver tumors, an incidence of 85 and 19% respectively.

Since a low protein diet gives a high incidence of tumors in azo dye carcinogenesis, the protein in the diet was lowered from 12 to 8% in the hope of increasing the incidence of tumors in female rats. However, these female rats had a lower tumor incidence (4%) than females on a 12% protein diet. This reduction of protein to 8% proved to be too low to support growth, as shown by poor weight gains. Thus low incidence of tumors in this group may be a starvation phenomenon. However, these rats lived to an age of 24 to 30 months and more.

Summary. Osborne-Mendel rats fed *p*-dimethylaminobenzene - 1-azo - 1-naphthalene (DAN) and *p*-dimethylaminoazobenzene (DAB) showed a higher incidence of liver tumors in males than in females. In DAN fed rats the respective incidence was 75 and 17% and in DAB fed rats 85 and 19%. Feeding higher doses of DAN (0.3%) to rats, failed to shorten the induction period for this compound. Rats fed *p*-dimethylaminobenzene-1-azo-2-naphthalene (DA-2-N) showed no significant difference in incidence of liver tumors between males (98%) and females (100%). However, tumors were much slower in appearing in females than in males, indicating relative resistance of the female liver to carcinogenesis induced by DA-2-N.

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Endogenous Respiration of Human Gingival Tissue. (24614)

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In the absence of marked anaerobic metabolism, it may be assumed that rate of oxygen consumption is a reasonable index of biochemical activity of a tissue. The value of 1.6 for endogenous QO_2 (dry weight) of human gingival tissue reported by Glickman *et al.*(1), using conventional Warburg Technic, appears to be quite low for a tissue which grows fast and heals rapidly. Laser(2) has shown that QO_2 of tissue in absence of added substrate decreases rapidly with time. A time-dependent decrease in respiration, together with delay in starting respiration trials, could well account for the low values previously obtained(1). To test such an explanation, we made use of a modified(3) Stern-Kirk microrespirometer and low incubation temperature (24°) to study time-dependence of endogenous respiration. Low temperature (under 8°) and speed were used in preparation of the slices(4).

Methods. Human gingival tissue, normal as judged by clinical and histologic standards, was obtained by surgical excision following block and/or local infiltration anaesthesia using Xylocaine HCl with adrenaline 1:100,000. The biopsy material was immediately placed into ice-embedded petri dish lined with filter paper, moistened with Ringer-phosphate solution(5). Slices 0.5 mm thick were prepared using Martin Slicer(6). To attain histologic uniformity, the biopsy was sliced in a direction perpendicular to mucosal surface. Slices were accumulated in cold Ringer-phosphate solution, and between 6 and 12 mg placed on lower shelf of platinum gold tissue rack. The rack was promptly lowered into the respiration chamber of respirometer which had been previously charged with 0.3 ml of Ringer-phosphate solution and equilibrated at 24°. A circle of filter paper to which was added 0.02 ml of 20% KOH was placed on upper shelf of rack. The lower shelf containing tissue slices was below surface of liquid, the upper shelf containing the CO_2 absorber was above the surface. The compensation chamber was charged in an identical manner except for tissue. The index droplet was centered, the instrument gassed with oxygen and needle valves closed. Readings were taken every 10 minutes for one hour. Time between excision of tissue and start of respiratory trials ranged from 15 to 30 minutes. Use of dental vibrator to shake microrespirometer, during previous trials established to our satisfaction that there was no rate-limiting factor in consumption of oxygen which could be removed by establishing turbulence in the bathing fluid. A portion from each biopsy sample, suitable for histologic examination, was placed immediately into 10% neutral formalin preparatory to processing and staining. Remainder of biopsy material (30-40 mg) was used to determine total nitrogen/unit wet weight of tissue. This value permitted calculation of QO_2 on the basis of wet weight of tissue(7). While wet wt. of larger pieces of tissue could be determined with reasonable accuracy, this was not so with small slices used in the respiratory trials. Instead, total nitrogen of contents of respiration chamber (tissue and fluid) was determined at end of trial, using the Kjeldahl-Nessler procedure outlined by Johnson(8) and the results multiplied by wet weight/total nitrogen obtained through analysis of larger piece of tissue.

Results. Studies were made on biopsy samples from 19 persons ranging in age 9-69 years. Values for total N/wet weight of 13 samples judged histologically, normal ranged from 0.0279 to 0.0355 with mean value 0.0316 ± .0036.*

Respiration trials at 24° were determined on 10 samples. QO_2 (wet) for individual sample averaged over 1 hour, varied from 0.265 to 0.446 with mean value 0.339 ± 0.068.* Mean QO_2 (N) was 10.67 ± 1.88.*

Stand. dev. $+\sqrt{\frac{\Sigma\,(x-\bar{x})^2}{n-1}}$, where $\bar{x} = \frac{\Sigma x}{n}$

The value for each 10-minute period, in terms of μl O_2/min/mg N, for the average for 10 samples is shown in Fig. 1 as a bar graph. The marked dependence of value of endogenous respiration on time is evident. Average QO_2 (N) calculated on the basis of first 10 minutes would amount to 14.1, while calculated value for last 10 minutes would be 8.1. The value calculated for data for first 10 minutes may not be the maximum attainable value since, due to scarcity of normal gingival tissue at time these studies were underway, it was necessary to include biopsy samples which had been infiltrated with local anaesthetic. We estimated from comparison with samples obtained by block anaesthetic only, that infiltration of the anaesthetic lowers the value by 10-15%.

To permit a rough comparison with data of Glickman *et al.*, 3 sets of tissue were determined at 24° and 38°. Ratios of QO_2 in favor of higher temperature were 4.8, 3.9, and 3.2. Average of these values (4.0) is somewhat higher than the value obtained by Fuhrman and Field(4) for the same temperature range using liver slices.

Discussion. The marked time-dependence of rate of endogenous respiration of human gingival tissue points out the need, more so than with some other tissues, to control rigidly the conditions under which respiratory trials are made. This would be especially so in any comparison of normal and pathologic gingiva. Undoubtedly, reliable and reproducible values would be obtained if the determinations were carried out in presence of added substrate and in presence of CO_2(2). If our value obtained at 24° is multiplied by a conservative factor of 3 to convert to 37.5°, and the QO_2 (dry) obtained by Glickman *et al.*(1) is divided by 5 to convert to wet weight basis, our value at 37.5° would be 1.0 and would be roughly 3 times their value (0.3) at approximately the same temperature.

After completion of this investigation, Schrader and Schrader(8) reported on respiration of gingival tissue. They obtained a QO_2 (wet) of 0.475 at 37.5°, a value about one-half as large as our conservatively estimated value (1.0) at 37.5°. These workers used slices 0.3 mm thick with air as the gas phase. Unfortunately, from the point of view of data in Fig. 1, they incubated their slices at 37.5° for 20-30 minutes before starting an hour-long respiratory trial.

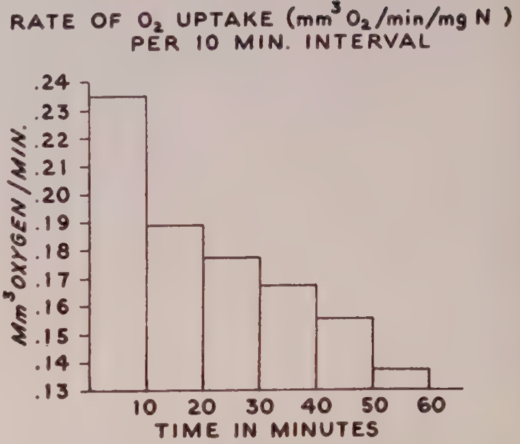

FIG. 1. Rate of endogenous oxygen uptake of normal gingiva for successive 10-min. intervals; composite of 10 studies at 24°.

Summary. Rate of endogenous respiration of human gingival tissue is markedly dependent upon time. However, reasonably high values may be obtained for endogenous QO_2 if speed and low temperatures are used in preparation of the slices. Respiratory studies on gingiva should be carried out in the presence of added substrate.

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Effect of Thymectomy on Development of Leukemia in C3H Mice Inoculated with Leukemic "Passage" Virus.* (24615)

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Thymectomy has been known to inhibit development of either spontaneous leukemia in Ak mice(1,2), or radiation-induced leukemia in strain C57 Black(3), or C3H(4). It appeared of interest to determine whether leukemia induced by inoculation of a transmissible, highly potent cell-free agent(5-7) could also be inhibited by removal of thymus.

Methods. Passage A strain of leukemic virus initially derived from spontaneous Ak leukemia(5), and then passed serially by cell-free inoculations of newborn mice(6,7), was used. In each experiment, filtrates (Selas 02) of 20% concentration, were prepared in the usual manner(6,7) from pooled leukemic organs removed from several individual C3H donors with primary, passage 12th to 14th, virus-induced leukemia. All mice were of the C3H, or foster nursed C3H(f) lines, both of Bittner substrain, bred in our laboratory by brother-to-sister mating. Incidence of spontaneous leukemia in untreated mice of our colonies of C3H and C3H(f) mice does not exceed 0.5%(7). Thymectomy was performed aseptically under combination of light, nembutal-ether anesthesia. After skin incision, the upper part of sternum was exposed, and the manubrium sterni split in middle line longitudinally (3 to 4 mm) with fine scissors. The fascia covering mediastinum was then gently lifted with an ophthalmic forceps, and a small opening (3 to 4 mm) made, to visualize the thymus. Both thymic lobes were then removed by gentle suction, using a small, glass tubing. The wound was closed superficially by 2 or 3 interrupted silk sutures, involving only the skin. Sufficient skill was gradually acquired to allow performing this simple operative procedure with mortality not exceeding 10 to 20%.

Results. Thymectomy in young adult C3H mice, followed by inoculation of leukemic virus. Young, adult C3H mice, approximately 4 to 6 weeks old, were divided into 2 groups. Brothers and sisters were equally divided, but not all animals were litter-mates. Mice of the experimental group were thymectomized. The other group was left untreated, as controls. After 3 to 7 days, animals of both groups were inoculated intraperitoneally (0.5 ml each) with leukemic filtrate. This experiment was based on the potency of newly developed passage A virus to induce leukemia, following inoculation into young adult C3H mice(7). It is evident from Table I that, thus far, all 39 thymectomized mice, which survived operative procedure and inoculation of virus, remained free from leukemia, whereas 44% of 41 controls, that had been inoculated simultaneously with the same filtrates, developed leukemia at

TABLE I. Thymectomy in Young Adult C3H Mice, Followed by Inoculation of Passage A Leukemic Filtrate.*

	Thymectomized mice†				Controls				
	No. of exp.	Sex	No. of mice inoc.	No. dev. leuk.	Sex	No. of mice inoc.	No. dev. leuk.	Leuk. inc., %	Avg age leuk. dev., mo
		♀	27	0	♀	27	11		
		♂	12	0	♂	14	7		
Total	3		39	0		41	18	44	7

* All filtrates (Selas 02) of 20% conc. A different filtrate was prepared in each experiment. Each mouse received 0.5 ml of filtrate i.p. Avg age at inoc., 60 days. Mice that died within 6 wk after inoculation were not included in table; this included operative mortality resulting from thymectomy.

† Time interval between thymectomy and inoculation of filtrate, 3 to 7 days.

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TABLE II. Inoculation of Passage A Leukemic Filtrate* into Suckling C3H Mice, Followed by Thymectomy.

	Thymectomized mice†					Litter-mate controls				
	No. of exp.	No. of litters inoc.	Sex	No. of mice inoc.	No. dev. leuk.	Sex	No. of mice inoc.	No. dev. leuk.	Leuk. inc., %	Avg age leuk. dev., mo
			♀	32	0	♀	39	38		
			♂	23	1	♂	39	30		
Total	20	38		55	1‡		78	68	87	2.7

* In each exp., different filtrate (20%, Selas 02) was prepared. 16 mice in thymectomized, and 18 in control group were inoculated s.c., when less than 16 hr old; all other mice in both groups inoculated i.p., when 1 to 8 days old (avg age at inoc., 3 days). Mice that died within 6 wk after inoculation were not included in table; this included immediate mortality resulting from thymectomy.

† Thymectomy was performed when inoculated mice were 3 to 6 wk old (avg age at thymectomy, 1 mo).

‡ 1 mouse developed palpable spleen at 2.7 mo of age, but is otherwise in good health.

7 months average age. Among 18 positive leukemic mice, 16 had a generalized leukemia, and in 2, on macroscopic examination, leukemia was limited to thymic lymphosarcomas. Although this experiment has not yet been terminated (average age of surviving mice 8 months at time of writing) the results thus far obtained are clear cut.

Inoculation of leukemic virus into suckling C3H mice, followed by thymectomy. C3H mice, less than 9 days old, were inoculated intraperitoneally with leukemic filtrate. After the mice reached 3 to 6 weeks of age, the litters were divided into one experimental, and a control group. Mice of the experimental group were then thymectomized (average age at thymectomy 1 month). Both groups were then observed for development of leukemia. It is evident from Table II that thymectomy inhibited development of leukemia, in essentially all animals, only 1 out of 55 mice developing a palpable spleen, and 1 developing definite leukemia. On the other hand, in the control group, 68 of 78 littermates developed leukemia (87%) thus far, mostly generalized (60) or limited on macroscopic examination to thymic lymphosarcomas(8). These experiments are still in progress (average age of surviving mice 4.5 months at time of writing); leukemia may still develop in some thymectomized mice, and will probably also develop in a few control animals. Even with these reservations, the results thus far obtained suggest inhibition of development of leukemia, following thymectomy, in mice inoculated with the leukemic agent.

Discussion. It was quite unexpected to observe that removal of thymus, either preceding, or following inoculation of leukemic passage A filtrates, inhibited development of leukemia in C3H mice. As an explanation, let us assume, as a working hypothesis, that the thymus represents the principal target organ, where the injected leukemogenic agent is stored, and multiplies. In the first series, there was no "target" organ available for the agent when the filtrate was injected, since mice had been thymectomized prior to inoculation. In the second series, removal of thymus in mice previously inoculated could have eliminated most of the virus presumably stored in that organ. The agent could have been stored in mice of both series also in other organs, such as spleen, liver, lymphoid nodes, etc. One might speculate that no disease resulted in most of such animals because a massive concentration of a potent agent may be needed in a particularly sensitive cell area, as may be available in the thymus, to assure sufficiently rapid replication, and eventual activation, of the agent. This would further imply that the initial focal activation of the disease would usually take place in the thymus, then disseminated rapidly. There is not sufficient evidence to imply, however, that all lymphatic leukemias in the mouse initially develop in the thymus. Other explanations therefore should also be considered; the role of thymus in development of leukemia may also be humoral, or otherwise activating the agent. Further studies are needed.

The second series, in which suckling C3H mice were first inoculated with leukemic virus, and then thymectomized, could be compared with the removal of thymus in young Ak mice; the latter carry naturally the same, or a very similar agent. In the injected C3H mice, the agent is introduced, by inoculation, into the suckling host, shortly after birth. In Ak mice, the agent is already present in the embryo, having been "vertically"(5) transmitted, *i.e.* acquired from parents. Both, injected C3H, and naturally infected Ak mice, carry therefore a potentially pathogenic leukemic agent. Irrespective of theoretical explanation, in both groups of animals, removal of thymus may inhibit development of leukemia.

That the thymus of young, normal Ak mice actually contains considerable quantities of the agent was evident from unpublished experiments we carried out, in which cell-free extracts prepared from normal thymic tissue aseptically removed from 4 to 6 week healthy Ak mice, were inoculated into newborn mice of the C3H strain, and induced leukemia. Other normal Ak organs, such as testes and ovaries, however, also carried the agent(8) suggesting that thymus is quite probably not the exclusive target area for storage of leukemic virus in the mouse.

It is true that C3H mice without injection of a leukemic virus will often develop leukemia following total body x-ray irradiation(4); this radiation-induced leukemia is apparently also caused by a transmissible virus, since it can be transmitted by filtrates(4,9,10). The agent apparently responsible for development of radiation-induced leukemia is of low potency, remaining latent, unless triggered by total body irradiation. This x-ray induced agent is apparently also stored in the thymus, since radiation-leukemia can also be prevented by thymectomy(3,4). Whether the Ak agent, causing leukemia following inoculation into C3H mice is identical with the latent agent, indigenous in normal C3H mice, and causing radiation-induced leukemia, remains to be determined; we believe that these are 2 distinct, though possibly related, leukemogenic agents. Whether acquired by inoculation, or by "vertical" transmission from host's parents, both are apparently stored principally in the thymus.

Thus, C3H mice in which thymus was removed during their first two months of life were resistant to leukemogenic action of either total body x-ray irradiation(4), or of transmissible virus known to induce otherwise leukemia in 90% of normal mice of the same inbred line(5-7).

Our results present a rather unusual example of preventing development of a transmissible, virus-caused disease, by surgical removal of a target organ, in an otherwise susceptible host.

It should be stressed, however, that although removal of thymus may have an inhibiting action on development of primary, virus-induced leukemia, it does not influence development of leukemia resulting from implantation of leukemic cells into a susceptible host. This was shown before(11) in Ak mice injected with Ak leukemic cells, and also observed in our laboratory on thymectomized young adult C3H mice inoculated with C3H leukemic cells (unpublished).

Summary. 1. Of 39 young and adult C3H mice, thymectomized, and a few days later inoculated intraperitoneally with passage A leukemic filtrate, none developed leukemia. Of 41 normal controls inoculated simultaneously with the same filtrate 18 (44%), developed leukemia at 7 months average age. 2. Of 55 suckling C3H mice inoculated with passage A leukemic filtrate when less than 9 days old, then 1 month later thymectomized, only 1 thus far developed a palpable spleen, and definite leukemia. Of 78 control litter mates inoculated simultaneously with the same filtrates, but not thymectomized, 68 (87%), developed leukemia at 2.7 months average age. 3. Thymectomy, either preceding, or following inoculation of leukemic passage A filtrate, inhibits development of leukemia in otherwise susceptible C3H mice.

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Inactivation by Plasma of ACTH-Releasing Property of C^{14} Labeled Bacterial Polysaccharides.*† (24616)

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A marked, but transient, elevation of plasma 17-hydroxycorticosteroids (17-OHCS) follows intravenous injection of a polysaccharide complex from *K. pneumoniae* into the guinea pig(1,2). The plasma 17-OHCS return to control, while abundant C^{14} labeled polysaccharide remains in blood plasma(2). During the first few hours after injection, the bacterial polysaccharide progressively loses capacity to evoke a 17-OHCS response(2). Such observations suggest that a physicochemical change may have occurred in the polysaccharide after its administration. It has been demonstrated that the polysaccharide will not deplete adrenal ascorbic acid or decrease adrenal cholesterol in hypophysectomized rats(3). Further, Rosenfeld(4) was unable to increase *in vitro* production of adrenal steroids by perfusing isolated adrenal glands with different amounts of polysaccharides. Hypercorticism following administration of polysaccharide must, therefore, be due to increased secretion of ACTH by the anterior pituitary gland. The present investigation concerns *in vivo* and *in vitro* binding of C^{14} labeled polysaccharide with plasma proteins in relation to ACTH releasing capacity and the haptenic property of the polysaccharide.

Materials and methods. The preparation of C^{14} labeled (alkaline-extracted, haptenic, non-lethal) polysaccharide complex from *K. pneumoniae* has been described(5). The haptenic quality of isotopically labeled polysaccharide in circulating plasma was evaluated by quantitative precipitin technic using anti-*Klebsiella* rabbit plasma inactivated at 56°C. The original labeled polysaccharide was employed as a reference standard. The optimal range of the precipitin reaction was 1 to 5 γ/ml of rabbit plasma. Controls consisted of polysaccharide in 0.85% NaCl with normal rabbit plasma, polysaccharide in circulating guinea pig plasma with normal rabbit plasma and polysaccharide-containing plasma with 0.85% NaCl. Guinea pigs of either sex, weighing 200 to 250 g were given a single injection by ear vein with 20 γ C^{14} labeled bacterial polysaccharide/100 g body weight. The labeled polysaccharide was given in 0.15 M NaCl, 0.1 ml/100 g body weight, and a control group was given 0.1 ml 0.15 M NaCl/100 g body weight. Animals in both groups were killed ½, 1, 3, and 6 hours afterwards, employing cervical clamp technic(2). The heparinized plasma obtained by cardiac puncture was immediately centrifuged and plasma removed. Plasma 17-OHCS were determined by the method of Eik-Nes(6). Previously we isolated cortisol in guinea pig plasma(4) and plasma levels of 17-OHCS in the guinea pig apparently reflect the concentration of cortisol in this fluid. Following cervical clamp technic(2), plasma levels of 17-OHCS in normal guinea pigs will range from 1 to 30 γ/100 ml with mean concentration of 21 γ/100 ml

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TABLE I. Mean Plasma Levels of 17-OHCS (γ/100 ml) in Guinea Pigs.

Treatment	Hr after treatment			
	½	1	3	6
.1 ml of .15 M NaCl/100 g body wt	27 ± 10.0* (3)†	35 ± 10.0 (2)	31 ± 9.8 (3)	21 ± 3.7 (3)
20 γ polysaccharide/100 g body wt in .15 N NaCl	126 ± 14.0 (4)	175 ± 39.0 (5)	234 ± 21.0 (5)	101 ± 12.4 (3)
20 γ polysaccharide/100 g body wt (6 hr plasma *in vitro*)	175 ± 3.7 (2)	185 ± 22.0 (3)	115 ± 23.0 (3)	94 ± 11.0 (3)
20 γ polysaccharide/100 g body wt (12 hr plasma *in vitro*)	85 ± 9.0 (4)	179 ± 18.0 (3)	154 ± 6.9 (3)	50 ± 9.5 (3)
20 γ polysaccharide/100 g body wt (6 hr plasma *in vivo*)	99 ± 3.0 (2)	125 ± 9.0 (3)	59 ± 10.0 (2)	29 ± 8.0 (2)
20 γ polysaccharide/100 g body wt (12 hr plasma *in vivo*)	106 ± 10.0 (2)	120 ± 11.0 (2)	39 ± 9.0 (2)	28 ± 7.0 (2)

* Stand. error of mean. † No. of animals tested.

and standard error of this mean of ± 5.2. Normal animals were given C^{14} labeled polysaccharide intravenously and exsanguinated 6 or 12 hours later. The heparinized blood was immediately centrifuged and plasma removed. An aliquant of plasma was used for determination of radioactive carbon and the remainder of plasma was injected into recipient guinea pigs by the ear vein, each animal receiving 20 γ of C^{14} labeled material/100 g body weight in less than 0.2 ml plasma/100 g body weight. These animals were killed ½, 1, 3, and 6 hours afterwards and plasma 17-OHCS determined. These experiments will be referred to as: 6 or 12 hours *in vitro* plasma. Pooled, heparinized plasma obtained from normal guinea pigs exsanguinated by cervical clamp technic was incubated at 39°C for 6 and 12 hours with labeled polysaccharide. All incubations were done immediately after blood was withdrawn. Normal animals were given 20 γ of C^{14} labeled material in 0.33 ml incubated plasma/100 g body weight, and plasma 17-OHCS were determined in these animals ½, 1, 3, and 6 hours after injection of the incubated plasma. These experiments will be referred to as: 6 and 12 hours *in vivo* plasma. Paper electrophoresis of guinea pig plasma from *in vivo* and *in vitro* experiments, containing 100 γ and 60 γ of C^{14} labeled material, respectively, was carried out in a Durrum type cell with veronal buffer, pH 8.6, ionic strength 0.1, potential gradient 6 volts/cm for 16 hours. Using a strip counter, the isotope material was localized on the electropherograms. Proteins were detected by densitometric measurements after staining with bromphenol blue.

Results. Intravenous administration of 20 γ of C^{14} labeled polysaccharide/100 g body weight in 0.15 M NaCl (Table I) in normal guinea pigs resulted in elevation of plasma 17-OHCS statistically greater than that seen following injection of saline alone, handling of animals and cervical clamp technic (p 1 hr. $<0.02>0.01$; p 2 hr. <0.01; p 3 hr <0.01; and p 6 hr. <0.01). The incubation of the bacterial polysaccharide with guinea pig plasma reduced the 17-OHCS evoking capacity of the polysaccharide. After the 6 hour *in vitro* incubation, only the 3 hour plasma 17-OHCS concentration differed significantly from that produced by the same amount of polysaccharide in saline (p 3 hr. <0.01; p 6 hr. >0.50), whereas the 12 hour incubation seemed to reduce further the ability of the polysaccharide to maintain high plasma concentration of 17-OHCS in the guinea pig (p 3 hr. <0.01; p 6 hr. <0.05, >0.02).

Although the polysaccharide in circulating plasma retained its haptenic properties (Table II), the *in vivo* polysaccharide was less capable of maintaining a high plasma concentration of 17-OHCS than similar quantities of labeled polysaccharide given in 0.15 M NaCl (p 3 hr. <0.01; p 6 hr. <0.02, >0.01).

TABLE II. Quantitative Precipitin Test with Rabbit Anti-*Klebsiella* Plasma and C^{14} Labeled Haptenic Klebsiella Polysaccharide.

	Ratio of C^{14} polysaccharide to antibody/ml*	C^{14} in precipitate, %
Polysaccharide in .15 M NaCl + anti-*K.* plasma	37.5	74.7
	18.7	77.9
	9.7	84.1
	4.7	91.9
Polysaccharide in .15 M NaCl + normal rabbit plasma	38.8	1.8
	19.5	3.2
Polysaccharide in normal guinea pig plasma + anti-*K.* plasma	18.7	68.4
	19.5	73.8
Polysaccharide in circulating guinea pig plasma + normal rabbit plasma	39.0	1.8
	19.5	1.6
Polysaccharide in circulating plasma *(in vivo)* + anti-*K.* plasma		
6 hr after inj.	35.6	64.3
	17.8	78.9
	8.9	84.6
	4.5	93.8
12 hr after inj.	12.4	64.0
	6.2	92.6
	3.1	98.0

* Total volume of precipitin test, 0.4 ml. Ratio of hapten solution to anti-*Klebsiella* plasma, 1:1.

Electrophoretic studies disclosed that the bacterial polysaccharide did not migrate in veronal buffer at pH 8.6 (Fig. 1). After 6 or 12 hours incubation with plasma, the polysaccharide migrated with the slower moving plasma proteins but not with albumin (Fig. 1). The polysaccharide in circulating plasma 6 or 12 hours after injection tended to migrate in proportion to amount of plasma protein and was especially prominent at the albumin peak (Fig. 1). Relatively less was found in α, β and γ globulins at 12 hours than at 6 hours.

Comment. Six and 12 hours after injection of C^{14} labeled polysaccharide, 28 and 20% respectively of the original radioactivity was found in the circulating plasma. The data suggest that the remaining bacterial polysaccharide had combined with plasma proteins and thereby lost a considerable amount of its capacity to evoke a response in plasma 17-OHCS, although the haptenic property remained. The nature of such binding seems unspecific, since the C^{14} labeled polysaccharide migrated electrophoretically with α, β, and γ globulins after an *in vitro* incubation, while the polysaccharide subjected to plasma *in vivo* tended to become uniformly distributed among plasma proteins.

Hegemann(7) and Goodale and co-workers (8) found that the pyrogenic effect of polysaccharides was reduced by incubation with fresh human plasma. Rall, *et al.*(9) made similar observations with rabbit serum. Landy and co-workers(10) reported that polysaccharides from *S. marcescens, S. typhosa* mouse sarcoma 37, and human erythrocytes lost the capacity to produce hemorrhagic necrosis in mouse sarcoma after 1 hour incubation at 37°C with human and guinea pig sera. Mouse serum had only a slight effect and rabbit serum had none. Incubation of polysaccharides with serum also reduced the preparatory capacity of the polysaccharide for the local Shwartzmann reaction and for the skin-necrotizing effect of epinephrine(10).

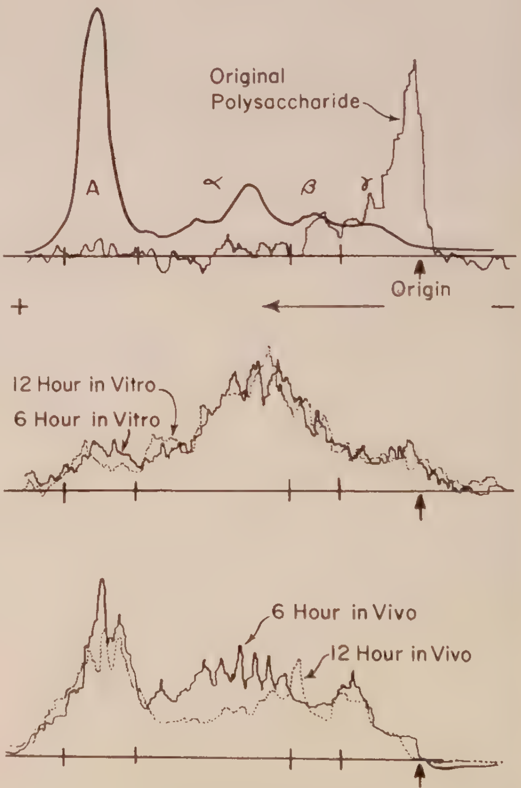

FIG. 1. Electrophoretic distribution of C^{14} labeled bacterial polysaccharide. A, albumin; α, α globulin; β, β globulin; γ, γ globulin.

Some attention has been drawn to the specific plasma protein components which may be effective in modifying various biologic responses of foreign polysaccharides. Landy, *et al.*(10) considered the properdin system to be unimportant in mouse sarcoma necrosis, while Cluff and Bennett(11) concluded that properdin associated with β globulin has an important role in inactivation of the pyrogenic effect with rabbit serum. Ho and Kass (12) found that Cohn's plasma fractions II + III, III and IV-1 reduced the lethal property of crude endotoxins in the rat. This may be related to modification of Ouchterlony precipitin bands with β globulin(13). Formation of polysaccharide-protein complexes is well known. Hyaluronic acid combines with various plasma proteins depending upon pH (14), heparin complexes with albumin(15), chondroitin sulfate binds with euglobulins (16), while bacterial polysaccharides, yeast zymosan and hog gastric mucin remove or inactivate properdin(17). Bernfeld *et al.*(18) reported that β-lipoglobulin and fibrogen form insoluble and soluble complexes with certain sulfated polysaccharides. These interactions with proteins involve relatively large quantities of polyanionic substances in contrast to our study with bacterial polysaccharide, in which the highest ratio by weight with protein is 1:300. Six and 12 hours after injection of the polysaccharide into the guinea pig, the ratio is approximately 1:1,000 and 1:1,500 respectively.

Apparently, increase in adrenocortical activity following polysaccharide administration is a result of ACTH release(3,4). ACTH may, therefore, be released from the hypophysis during the first moments after injection of polysaccharide and before protein-polysaccharide combination has occurred. Since the polysaccharide loses some of its ability to influence the pituitary-adrenal system, both by incubation with guinea pig plasma and during its circulation in the guinea pig, the combination between the polysaccharide and the plasma proteins may result either in blocking the part of the polysaccharide responsible for ACTH release or also making the polysaccharide less toxic and hence, less of a "stress" for the animal. The pathways through which the bacterial polysaccharide produces a stimulation of the pituitary-adrenal axis in the guinea pig are, however, not known.

Summary. A non-lethal, haptenic polysaccharide from *K. pneumoniae,* given intravenously to guinea pigs, produces a marked, but transient, elevation in plasma 17-hydroxycorticosteroids. Incubation of the polysaccharide with normal guinea pig plasma partially inactivates this response. The polysaccharide remaining in the circulating plasma 6 and 12 hours after intravenous injection shows a greater loss of this ACTH releasing property. Electrophoretic studies indicate that the polysaccharide is bound to plasma proteins, but there are marked differences in *in vitro* and *in vivo* binding with albumin. The bound polysaccharide, however, retains its haptenic qualities. These findings suggest that the bacterial polysaccharide undergoes little, if any, degradation but loses its 17-OHCS evoking capacity by combining with plasma proteins.

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Hypothermia: IV. Study of Hypothermia Induction Time with Various Pharmacological Agents. (24617)

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Hypothermia is of interest from its practical application(1) as well as from the observed basic physiologic alterations. At present, most of the interest in hypothermia concerns its application to cardiovascular surgery and neurosurgery(2). McQuiston(3) reported cases of 25 children subjected to various cardiac operations under hypothermia. Bigelow (4) and Cookson(5) performed dog experiments in which they occluded both vena cavae during hypothermia thus giving the surgeon a bloodless field for intra-cardiac surgery. Application of the principle of hypothermia for diminishing vital tissue oxygen requirements in patients undergong cardiac surgery was successful by Swan and his associates(6). Interest in hypothermia was aroused also by the concept of "artificial hibernation" developed by Laborit and Hugenard(7). They achieved their "artificial hibernation" through use of a "lytic cocktail" (a pharmacological mixture containing chlorpromazine, promethazine, and meperidine). Their major interest was to potentiate general anesthesia, and counteract the shock and other undesirable reactions to surgical or traumatic stress, but it was also noted that with supplementary body cooling, hypothermia could be obtained easily. Physiological alterations resulting from the lytic cocktail in patients undergoing surgery have been summarized by Shackman *et al.*(8). The effectiveness of the lytic cocktail for inducing hypothermia has been tested by Dundee *et al.* (9), who also concluded that muscle relaxants were equally effective. Moyer *et al.*(10) compared the effects of agents which block the sympathetic nervous system on hypothermia induction time. The rationale for this study was that the autonomic response to hypothermia supposedly provides a barrier to hypothermia induction. Adrenergic blocking agents (chlorpromazine and Hydergine)* which also possess central effects, Dibenzyline, an adrenergic blocking agent with minimal central effects, and mecamylamine (some central effect) were compared. They concluded that sympathetic nervous system depression *per se* did not affect hypothermia induction time and that chlorpromazine and mecamylamine (less effect than the former) were the only effective agents which reduced induction time due, apparently, to the anesthetic potentiating effect of these drugs. Our study was a further attempt to compare the effect of various pharmacologic agents on hypothermia induction time and to elucidate the locus of action of some of these agents. Chlorpromazine, the lytic cocktail containing chlorpromazine, promethazine, and meperidine, and d-tubocurarine were studied. Chlorpromazine and the lytic cocktail (promethazine, meperidine, and chlorpromazine) were compared to see how effective chlorpromazine plus adjunctive agents were, in contrast to chlorpromazine alone. Peripheral blockade of corticospinal pathways at the neuro-muscular junction by use of d-tubocurarine was studied to localize sites of action of effective agents. We were also interested in the effect of anesthesia alone compared to the more diverse pharmacological action of the various drugs.

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* Equal mixtures of dihydroergocornine, dihydroergokyptine and dihydroergocrystine.

TABLE I. T/W* Values in 12 Dogs.

Group	I	II	III	IV
	Hypothermia alone (no drug, but anesthesia)	Hypothermia with		
		Chlorpromazine	Lytic cocktail	Curare
	11.2	7.6	12.0	11.8
	12.4	9.5	6.3	9.7
	9.5	7.3	8.0	9.0
	11.5	7.9	7.3	9.0
	10.9	9.2	8.2	9.5
	11.6	6.9	8.6	10.0
	11.9	10.2	9.4	9.0
	11.9	10.4	8.7	7.0
	11.7	9.2	7.7	12.2
	10.8	7.3	10.5	11.9
	8.5	9.2		
	10.5			
Mean values	11.0	8.6	8.7	9.9
P values less than		.001	.01	.1
P " comparing Group II & IV				.05

$$* \, T/W = \frac{\text{Time in min. to reduce dog's temp. to } 80°\text{F}}{\text{Dog's wt in kg}}.$$

Methods and materials. The method for determining hypothermic induction time was the same as that used by Moyer *et al.*(10). Unselected mongrel dogs, varying in weight from 8 to 16 kg were used. A surface cooling refrigerated blanket was the hypothermia apparatus used. The blanket was precooled to 20°F before being applied. The dogs were anesthetized with 30 mg/kg of sodium pentobarbital given intravenously. The hair was shaved from thorax and abdomen to prevent variability due to insulating effects of different thicknesses of hair. The dogs were given the drug, then placed on the blanket immediately. Induction time was the time required to cool the dogs to 80°F as measured by rectal thermometer. During induction, shivering response, depth of anesthesia, interval temperatures, the electrocardiogram and respiration were observed. When respiration became significantly depressed, the dogs were maintained on an artificial respirator. All drugs were given intravenously, in the following dosages: Group I, 12 dogs, Control; Group II, 11 dogs, Chlorpromazine 5 mg/kg; Group III, 10 dogs, lytic cocktail, meperidine 6.6 mg/kg, promethazine 3.3 mg/kg, chlorpromazine 5 mg/kg; Group IV, 10 dogs, d-tubocurarine, .15 mg every 40 min. Dosages of the lytic cocktail, with exception of that of chlorpromazine which for purposes of comparison could not be greater than that of Group II dogs, were based on the method of Laborit. Without pentobarbital, 3 dogs were given varying dosages of chlorpromazine (from 30 to 70 mg/kg) alone and their response noted.

Results. The results are presented in Table I. Group I consists of 12 dogs in which hypothermia induction times were observed with only pentobarbital anesthesia(10). In Groups II, III and IV, induction times were determined witsh pentobarbital plus chlorpromazine, the "lytic cocktail," and d-tubocurarine respectively. About 50% of dogs in Group I showed a significant amount of shivering. None of the dogs in Groups II, III or IV showed any shivering. To compare individual dogs or groups of dogs, an arbitrary ratio of T/W (induction time/weight of dog in kg) was set up. T/W ratio for dogs with hypothermia alone was 11 as compared to Group II (chlorpromazine) T/W of 8.6 ($p<0.001$) and Group III (lytic cocktail) T/W of 8.7 ($p<0.01$). In comparing Group I and Group IV, the p value was >0.1, the T/W of the latter being 9.9. A significant difference existed between Group II (T/W 8.6) and Group IV (T/W 9.9); p value was <0.05.

Of 3 dogs which received chlorpromazine without concomitant pentobarbital, none was sufficiently anesthetized to be kept on the cooling blanket for any length of time. All dogs had a maximal shivering response. At doses of 30 mg/kg and 40 mg/kg 2 dogs showed no temperature reduction and anesthesia was too light for the dogs to be kept on the blanket. The third dog received 70 mg/kg, remained on blanket 60 minutes, and underwent temperature reduction from 100° to 93°F. On Group II dogs (chlorpromazine 5 mg/kg and pentobarbital) the average temperature level after 60 minutes was 87.8°.

Discussion. To be a useful procedure, hypothermia must be induced safely and efficiently. The time factor is important because the longer a patient is submitted to hypothermia, the greater the chance of developing complications (ventricular fibrillation, hemorrhagic tendency)(2). When induction time is shorter it suggests that the animal is not fully utilizing his defense mechanisms against temperature reduction, shivering, vasoconstriction, and pilo-erection(12). Our experiment does not show whether or not these defense mechanisms (autonomic nervous system and/or hormonal response) are harmful to the patient, but we did show that both lytic cocktail and chlorpromazine reduce induction time. There was no significant difference betwen lytic cocktail and chlorpromazine indicating that giving other agents in the lytic cocktail had no additional effect, as previously suggested(9). The difference between induction times of hypothermia alone and d-tubocurarine was not statistically significant; however, the curare preparation did prevent shivering, suggesting that neuromuscular blockade did not alter induction of hypothermia significantly. Average T/W ratio was 1.1 units less than that of the controls (Group I).

Chlorpromazine, combined with pentobarbital anesthesia was the most effective hypothermia inductant studied. The mechanism of action is of interest. Physiologic thermoregulation is performed by a forebrain center(12) which controls several peripheral mechanisms, by which an animal combats cold. One of these mechanisms of heat gain, shivering, has been shown to involve higher pathways and does not occur as a simple spinal reflex. The hypothalamus seems to contain the thermoregulatory center(12) which is divided into a rostral area which prevents overheating, and a caudal area preventing heat loss. This center is probably stimulated in 2 ways(13)—reflexly from the skin and by the temperature of the blood flowing through the center. Somewhere along these pathways effective agents make induction of hypothermia easier.

Moyer and his associates showed that a potent adrenergic blocker (Dibenzyline) was not an effective inductant; supposedly this would have blocked the vasoconstrictor response. We showed that there was a significant difference between effect of chlorpromazine (Group II) and d-tubocurarine (Group IV) and also that there was not an impressive difference between control (Group I) and curare (Group IV). However, both the d-tubocurarine and the chlorpromazine (with pentobarbital) eliminated shivering indicating that abolition of shivering is not all-important to the chlorpromazine effect. From our experience with the 3 dogs which received chlorpromazine without pentobarbital, it is evident that the latter is essential to the chlorpromazine effect. Also chlorpromazine (with pentobarbital) does have a marked effect when compared with pentobarbital alone. This anesthetic potentiating effect of chlorpromazine when combined with pentobarbital appears to be the primary factor by which induction time is reduced. The results gained by use of peripherally acting agents lead to a more complete understanding of the action of chlorpromazine as follows: 1) Prevention of shivering is not the only action of chlorpromazine, because this also is eliminated by d-tubocurarine. 2) The adrenergic blocking action of chlorpromazine is not significant enough to account for its effect on induction time. 3) The effect of chlorpromazine in accelerating hypothermia induction time is apparently central where it probably provides an effective block of heat gain mechanisms.

Summary and conclusions. Thirty-one dogs were cooled down to 80°F and the ability of various pharmacologic agents to facilitate hypothermia induction was observed. 1) Chlorpromazine and the "lytic cocktail" accelerate hypothermia induction time. 2) Chlorpromazine is apparently the only effective agent in the "lytic cocktail." 3) Chlorpromazine accelerates hypothermia induction by a central action. 4) Pentobarbital anesthesia is essential for production of the chlorpromazine effect.

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Hematology in Vit. B$_{12}$ Deficient Newborn Rat.* (24618)

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With isolation of Vit. B$_{12}$(1,2), it was shown that this vitamin fulfilled the role of Castle's extrinsic factor as well as that of the hemopoietic factor in liver(3). A number of investigators(4-7) reported the effect of Vit. B$_{12}$ deficiency on blood of animals with rather wide variations in effects produced among different species. This paper includes results of blood and related tissue studies of newborn rats littered to Vit. B$_{12}$-depleted dams.

Methods. Newborn offspring of Vit. B$_{12}$-deficient and Vit. B$_{12}$-supplemented dams were used. Dams were depleted of Vit. B$_{12}$ by maintaining them on soybean meal-glucose type diet from time of weaning. Methods employed for production of the deficiency and for histological studies were described previously(8). Blood was obtained by decapitation and hemoglobin was determined by direct photometric method(9). A Hellige-Diller colorimeter, calibrated with a sample of blood whose hemoglobin content was calculated from its iron analysis, was used for measurement. Red cell, leucocyte and differential counts on blood obtained as described above, were made by standard procedures. Erythrocytes were examined for sickling by mounting a small drop of blood with a drop of fresh, 1% aqueous solution of sodium bisulfite under coverslip and examining microscopically after 5 minutes. Hematocrits were determined in Van Allen hematocrit tubes using 1.3% sodium oxalate as diluent.

Results. Table I shows that a significant difference in blood picture existed between controls and Vit. B$_{12}$ deficient newborn rats. Hemoglobin, hematocrit values and erythrocyte counts were considerably decreased in the deficient rats. Leukocyte counts were reduced to about one-half that of controls and the differential counts showed this difference to be manifest mainly in percentage of mature neutrophils. Neutrophilic granulocytes appeared unable to mature properly leaving more cells in the myelocytic and metamyelocytic stages.

Erythrocyte concentration was reduced significantly in peripheral blood and large numbers of nucleated red cells were present in the deficient animals. Peripheral blood films showed polychromatophilia, anisocytosis, poikilocytosis, smudge cells, and objects resembling degenerate erythrocytes (Fig. 1). Sickling mounts indicated that these were not true sickle cells.

Bone sections showed that the bone was poorly developed. There was a lack of normal calcification and a failure of normal endochondral bone growth and remodeling sequences. Osteoblasts were reduced in number and those present appeared less active than controls. Both cartilage cells and osteoblasts contained very little glycogen compared to controls. Clumps of cartilage cells were observed in the bony collar, and in many cases tongues of nucleated cartilaginous cells extended down into the marrow cavity from the epiphyseal plate (Figs. 2 and 3). Bone marrow from deficient animals showed active hemopoiesis and a 2- to 3-fold higher concentration of pro-erythrocytic elements, a large portion of which were pronormoblasts (Figs. 4

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TABLE I. Hematology of Newborn Rats.

Vit. B_{12} status of dam	Hemoglobin, g/100 ml	Hematocrit, %	RBC $\times 10^6/mm^3$	WBC $\times 10^3/mm^3$	Lymphocytes, %	Myelocytes, %	Metamyelocytes, %	Neutrophils, %
Supplemented	10.32 ± .63*(56)†	37.51 ± 3.5 (56)	2.408 ± .19 (30)	4.133 ± .83 (36)	26.1	25.9	25.6	22.4
Deficient	8.34 ±1.26 (94)	28.43 ± 3.7 (94)	1.755 ± .24 (67)	2.689 ± .97 (57)	37.2	23.2	24.9	14.7
Significance of diff. (P)‡	<0.01	<0.01	<0.01	<0.01				

	Mean corpuscular vol (μ^3)	Mean corpuscular hemoglobin ($\mu\mu g$)	Mean corpuscular hemoglobin conc. (%)
Supplemented	148.6	42.5	28.6
Deficient	160.1	45.0	30.9

* ± stand. dev.

† Numbers in parentheses indicate number of observations.

‡ P determined by Fisher "t" test.

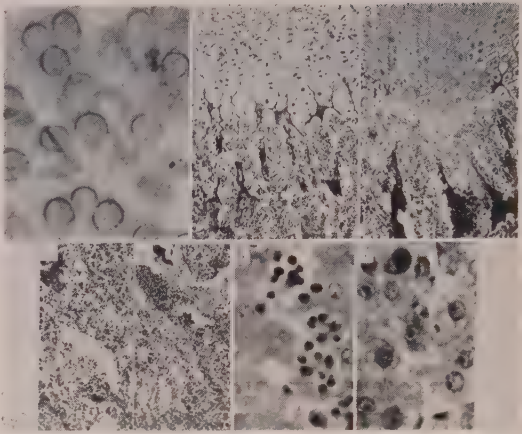

FIG. 1. Red blood cell smear from newborn B_{12} deficient rat showing objects resembling sickle cells. Some were irregularly rounded with pronounced vacuolations. Wright's stain, × 215.

FIG. 2. Epiphyseal area from femur of a control newborn rat. Note orderly arrangement of cartilage cells and endochondral bone growth. H. & E. stain, × 30.

FIG. 3. Epiphyseal area from femur of newborn vit B_{12} deficient rat showing irregular shaft of cartilage extending into marrow cavity. Many of these cartilage cells contain nuclei. H. & E. stain, × 30.

FIG. 4. Bone marrow from newborn control rat showing normal cellularity. H. and E. stain, × 30.

FIG. 5. Bone marrow from newborn vit B_{12} deficient rat showing increased cellularity. Much of the increase was due to immature erythroid and myeloid elements. H. & E. stain, × 30.

FIG. 6. Liver section from control newborn rat showing extramedullary hemopoiesis within sinusoids. H. & E. stain, × 267.

FIG. 7. Liver section from vit B_{12} deficient newborn rat showing lack of erythropoiesis, presence of cytoplasmic lipide accumulations, and large, irregular clumps of nuclear chromatin. H. & E. stain, × 267.

and 5). The granulocytic elements contained pale, vacuolated nuclei.

The liver is a part of the erythropoietic system in the infant rat, and for this reason the organ was studied histologically along with blood. Liver sinusoids showed vascular blocks and were plugged with fatty material and cellular debris. The nuclei showed margination and clumping of deeply staining basophilic chromatin material. The cytoplasm of cells was distended with lipid material. There was a marked reduction in hemopoietic activity with only a few erythroblastic elements scattered about in the tissue (Figs. 6 and 7). These elements were densely chromatic and many were pyknotic.

Discussion. Anemia, leukopenia and granulocytopenia observed in Vit. B_{12} deficient animals was similar to that reported by Borson *et al.*(7). The fact that the greatest change in leukocytes was in mature neutrophils, suggests that neutrophilic granulocytes were unable to mature properly in absence of Vit. B_{12}. The observation on peripheral blood films was supported by bone marrow studies. Granulocytic myelocytes contained pale, vacuolated nuclei deficient in nuclear chromatin material.

The presence of large numbers of nucleated erythrocytes in peripheral blood film of deficient animals indicated release of these cells from marrow before they were matured. This finding and the anemia which was present in deficient animals suggests that deficiency of Vit. B_{12} has an effect on the newborn rat similar to blood dyscrasia in pernicious anemia of man. Sickle-shaped objects were not true sickle cells; they may have been fragments of red blood cells which had undergone degeneration as a result of increased fragility.

The changes observed in liver agree with those reported by Jones *et al.*(10). The reduction in hemopoietic elements in liver of deficient rats no doubt contributed to the anemia, which was primarily the result of malfunctioning bone marrow. Accumulation of fatty substances in the hepatic cells suggests that lipid metabolism is deranged as a result of Vit. B_{12} deficiency.

Reduced calcification of bone as well as decreased osteoblastic activity may be related to reduced phosphatase activity in Vit. B_{12} deficient bone(11). Immaturity of bone as well as of other tissues is related to a more generalized failure of cells to undergo normal cell division and growth.

Summary. Vit. B_{12} deficiency in the dam resulted in newborn rats with anemia, leukopenia and granulocytopenia. The liver showed a marked reduction in hemopoietic elements, vascular blocks, and an accumulation of lipide in the hepatic cells. The femur of Vit. B_{12} deficient rat showed abnormal patterns of growth and bone marrow contained increased concentration of hemopoietic elements. However, most cells appeared unable to complete maturation.

The above data are taken in part from thesis presented by P. M. Newberne in partial fulfillment of requirements for Ph.D. degree, University of Missouri.

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Incorporation of Glucuronolactone-6-C^{14} into Connective Tissue Polysaccharides.* (24619)

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In view of recent findings that D-glucuronolactone serves as a precursor for synthesis of L-gulonic acid(1), L-ascorbic acid(1-5) and L-xylulose(6,7), it was considered of importance to investigate whether D-glucuronolactone could be incorporated into acid polysaccharides (APS) of connective tissues. For this reason large doses of D-glucuronolactone-6-C^{14} were administered over a period of 2½ days to actively growing rats. Experiments were also carried out in which this labeled compound was administered to guinea pigs after subcutaneous injection of carrageenin to stimulate formation of large amounts of connective tissue(8,9). The APS fraction was isolated from samples of connective tissue obtained from these animals and their C^{14} content determined. Results showed no significant incorporation of glucuronolactone into APS fraction of connective tissues.

Materials and methods. Albino rats of the Wistar strain, 25-30 days old, weighing 50-70 g were used. Thirty mg of D-glucuronolactone-6-C^{14} (1 μc/mg) was injected intraperitoneally in divided doses of 5 μc twice a day for 3 days and animals sacrificed 6 hours after last dose. The skins were shaved, removed, freed of fat and muscle, minced, defatted and dehydrated with acetone and ether and then dried. A pooled sample of tail tendon, rib cartilage, trachea and nasal septum was also obtained from these rats and treated the same as the skins. Guinea pigs weighing 400-500 g were injected subcutaneously with 5 ml of a solution containing 50 mg carrageenin. On fifth and sixth days after receiving carrageenin, D-glucuronolactone-6-C^{14} (20 μc) was injected intraperitoneally in doses of 5 μc twice daily for 2 days. Six hours after last dose, animals were sacrificed and newly formed connective tissue was removed from abdominal wall, freed of extraneous material, homogenized in acetone, and a homogeneous powder was obtained after removal of acetone. Two different methods were used for extraction and isolation of APS from connective tissues. (a) *Chemical.* Dried tissues were extracted with 1 N NaOH (15 ml/g) at 70° for 30 minutes, and the resulting solution was neutralized with acetic acid and clarified by centrifugation(10). From the supernatant, APS were precipitated with cetyltrimethylammonium bromide (cetavlon)(11), or benzidine dihydrochloride(12). (b) *Enzymatic.* Weighed samples of dried connective tissues were digested by trypsin, dialyzed and the APS were precipitated with acetone(13). The precipitate was washed with alcohol and ether-dried, and analyzed for radioactivity. The isolated APS were dissolved in water, aliquots were plated and radioactivity determined by a windowless gas-flow counter. The methods for C^{14} assay in tissues reported by Burns *et al.*(14) were used for determination of radioactivity in skin and granuloma tissue.

Results. Results in Table I show per cent incorporation of C^{14} from labeled D-glucuronolactone into the APS fraction of connective tissues. The small amounts of C^{14} found in these tissues could come from fixation of $C^{14}O_2$ formed in metabolism of D-glucuronolactone. Considerable radioactivity was found in the supernatant fraction obtained from cetavlon precipitation and in the dialysate of enzymatic digestion. These findings suggest that D-glucuronolactone was utilized for formation of labeled compounds of small molecular weight. Similar results were obtained in the APS fraction of the carrageenin induced connective tissue of guinea pigs (Table I). In this newly formed, highly metabolizing tissue, about 1% of administered dose of radioactive D-glucuronolactone was present, but no significant incorporation into the APS fraction was found as determined by both enzymatic and chemical methods. Two

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TABLE I. C^{14} in APS Fraction after Administration of Glucuronolactone-6-C^{14}.

Exp.	Tissue	Dry wt tissue, g	Method of isolation	Total C^{14}, cpm	% of dose
1*	Skin	3.54	Cetavlon	5660	.011
			Enzymatic	9200	.02
	Pooled tissue	.28	Cetavlon	5200	.01
2†	Skin	1.3	"	1580	.01
			Enzymatic	2400	.015
	Pooled tissue	.075	Cetavlon	1650	.01
3†	Granuloma	1.5	"	1500	.01
			Enzymatic	3000	.02
4†	"	1.8	"	4000	.025

* Tissues from 2 rats representing 60 μc total dose.

† One animal/exp. receiving 5 μc twice a day for 2 days.

control experiments carried out under the same conditions with glucose-6-C^{14}, showed a .1% incorporation of C^{14} into APS fraction of granuloma tissue and skin.

Discussion. The C-6 labeled glucuronolactone was used in these experiments instead of the uniformly labeled compound, to eliminate non-specific incorporation from carbons 1-5; studies have shown that the C-6 of glucuronolactone forms CO_2 but carbons 1-5 go to glucose(15).

Newly formed connective tissue in the actively growing rat and the carrageenin induced granuloma rich in acid polysaccharides has provided a good method of studying whether D-glucuronolactone is utilized for formation of APS. Although D-glucuronolactone is utilized for formation of L-gulonic and L-ascorbic acids to a greater extent than glucose(1-5), the .01% conversion of this compound, or 1/10 that of glucose, into APS found in our studies appears to be insignificant. These results and reports of other investigators that D-glucuronolactone does not serve as a precursor of menthol and borneol glucuronides(16,17) suggest that there may be a different pathway of formation of D-glucuronic acid for synthesis of glucuronides and tissue APS, than that for L-gulonic acid and L-ascorbic acid.

Summary. D-glucuronolactone-6-C^{14} was administered to actively growing rats to study its incorporation into connective tissue acid polysaccharides. The same compound was administered to guinea pigs after injection of carrageenin which stimulates formation of new connective tissue. No significant incorporation of glucuronolactone into the APS fraction of connective tissues was found.

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Translocation of Radioactive Silver Compounds from Site of Injection into Organs.* (24620)

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We have outlined previously(1) a method for studying comparatively translocation of various radioisotopes in the mouse body. The method implied: a) subcutaneous injection of radioactive material into tails of animals, b) amputation of injected tails at various intervals, c) simultaneous autopsies in all animals, and d) counts of radioactivity in tails, blood, and some vital organs obtained by autopsies. Recently, we have applied this method in distribution studies on mice with various compounds of the same isotope, radioactive silver Ag^{111}, to provide some information on relationship between fate of an isotope in the body and physico-chemical properties of the compound into which this isotope is incorporated. Moreover, in another series of mice, some Ag^{111} compounds were injected by the same route (subcutaneously) but into a topographically different area of skin (scalp) and processed analogously (by autopsies for radioactivity). This experiment was intended to compare the effects of biological factors (topography of site of injection) on distribution.

Materials and methods. Swiss Albino male mice of 25 to 27 g weight were used. Radioactive Ag^{111} separated from palladium target and purified was obtained from Oak Ridge A.E.C. Laboratories and transformed into oxide, iodide, chloride, phosphate, and lactate.† Insoluble material was washed several times with distilled water, suspended at a ratio of 5 mc/10 cc and vigorously shaken before injection; 0.1 ml of solution or suspension was injected. A 25-gauge needle was used to inject the preparation into a thin layer of connective tissue on ventral side of the tail or subcutaneously over the scalp. Autopsies and radioactive counts were described previously (1). For interpretation comparable to previous work(1,3) we calculated the radioactivity for each mouse using radioactivity of heart blood (0.3 ml) as a standard unit. Thus, we have recorded the average pattern for each organ in each group of mice as the "organ/blood" ratio at various stages of translocation. For comparison of blood radioactivity in various groups of animals in the same experiment (series), all values in a particular series are ratios of the average radioactivity in the blood of the animals in Group 1. It should be noted that carrier-free Ag^{111} is a new product and we were unable to obtain precise information on composition of its solids; thus, in using it, we were able to give only doses of radioactivity, but not chemical doses and we realize the necessity of additional information for interpretation of our results. Nevertheless, our scouting experiments provide sufficient preliminary information for comparative trial of these compounds in tumor-bearing animals.

Results. The calculated ratios were arranged in Tables according to site of injection (Table I - Tail, Table II - Scalp) for various compounds (series) and in Table I, for various intervals (groups) between injection of radioactive compound and removal of injected tissue (*i.e.,* tail amputation). Thus, in each Table, each vertical column shows the uptake of various Ag^{111} compounds by the same organ, while each horizontal line illustrates pattern of distribution of each compound between various organs. Table II compares only with the 24 hours group in Table I.

Series A. Table I shows that injected Ag^{111} nitrate was stored in the tail during 10 minutes after injection, but within the following 50 minutes it was mostly translocated into the liver and significantly into the kidney. After 24 hours, the radioactivity pick-up in these organs remained nearly constant; blood radioactivity, which was temporarily in-

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creased, returned closely to its initial level. Distribution of the same compound 24 hours after injection into the scalp roughly resembled its pattern recorded 30 minutes after tail injection; in other terms, translocation from both sites followed the same pattern but was considerably delayed from the scalp.

Series B. During the first hour, Ag^{111} lactate was much lower than Ag^{111} nitrate in the tail and in blood whereas localization was higher in lung, kidney, and spleen. Moreover, most translocation from the tail occurred with-

TABLE I. Spread of Various Ag^{111} Compounds from Site of Injection (Tail) into Blood and Visceral Organs at Various Intervals before Removal of Injected Tissue (Tail Amputation).

Compound of Ag^{111}		Time between Ag^{111} inj. into tail and tail amputation	Radioactivity content of inj. tissue (tail) and of visceral organs expressed as ratios of avg radioactivity in same group					Comparison of blood radioactivity in different groups of same series with Group 1 as 1
Series (letters)	Groups (numbers)		Tail	Lung	Kidney	Spleen	Liver	
A. Ag^{111} nitrate								
	1	1 to 5 min.	478.4	.9	1.2	1.0	1.4	1.0
	2	10	464.1	1.2	1.1	1.1	1.2	1.6
	3	30	121.5	1.3	1.3	1.8	4.1	2.9
	4	60	96.5	2.1	1.8	1.5	8.1	3.3
	5	3 hr	84.3	1.8	2.1	1.2	7.3	2.9
	6	24	66.1	1.2	2.1	1.1	9.3	1.6
	7	48	62.1	1.6	2.2	1.1	9.8	1.3
	8	72	67.0	1.6	2.2	1.2	9.1	1.2
B. Ag^{111} lactate								
	1	1 to 5 min.	241.1	3.2	1.8	1.6	4.1	1.0
	2	10	189.4	3.1	1.3	1.4	5.9	1.3
	3	30	79.8	2.1	1.0	1.4	6.4	1.4
	4	60	49.6	1.8	1.0	1.4	8.2	1.3
	5	3 hr	41.5	1.0	1.3	1.2	10.1	2.2
	6	24	27.1	1.2	1.1	1.8	12.1	2.6
	7	48	15.9	1.7	1.4	1.5	12.3	1.2
	8	72	11.1	1.9	1.9	1.2	11.1	1.2
C. Ag^{111} oxide								
	1	1 to 5 min.	623	.9	1.0	1.2	1.2	1.0
	2	10	487	2.2	1.6	1.1	1.4	1.3
	3	30	431	1.0	.9	1.0	2.1	2.0
	4	60	390	1.1	.8	3.1	4.4	1.9
	5	3 hr	331	1.5	.9	2.2	5.9	1.3
	6	24	287	1.8	1.4	2.3	8.8	1.3
	7	48	153	3.2	1.2	2.8	9.2	1.6
	8	72	151	5.2	1.1	3.9	9.8	1.3
D. Ag^{111} chloride								
	1	1 to 5 min.	970	2.1	1.5	3.0	1.3	1.1
	2	10	790	3.2	2.8	5.3	2.1	1.3
	3	30	700	3.1	2.0	2.3	2.2	1.6
	4	60	611	2.4	2.1	4.7	4.2	1.6
	5	3 hr	510	2.5	1.4	4.8	6.4	1.2
	6	24	360	2.6	1.8	4.3	9.1	1.1
	7	48	235	6.1	2.0	4.7	9.7	1.1
	8	72	191.8	2.8	1.3	5.1	10.3	1.9
E. Ag^{111} iodide								
	1	1 to 5 min.	183	1.6	1.7	1.7	2.5	1.0
	2	10	178	2.1	1.3	1.6	3.5	1.1
	3	30	103	1.7	1.2	1.6	4.3	1.3
	4	60	81.9	1.8	1.8	1.3	4.3	1.5
	5	3 hr	61.8	1.8	1.7	1.5	5.2	1.5
	6	24	38.2	1.7	1.7	1.0	4.1	1.7
	7	48	32.2	1.6	1.7	1.1	4.4	1.4
	8	72	30.6	1.6	1.5	1.1	3.3	1.5

Ratio for each tissue is avg of 15 mice in each group.

TABLE II. Spread within 24 Hours in Body of Mouse of Silver Compounds Injected into the Scalp.

Series and compounds		Tissues and organs						Comparison of blood radioactivity in different series with Series A (in Table I) as 1
		Scalp	Periost. and bone (skull)	Lung	Kidneys	Spleen	Liver	
A.	Ag^{111} nitrate	138.0	31.4	1.4	1.9	1.5	7.1	3.1
B.	lactate	65.2	11.2	1.7	1.3	1.4	8.1	2.6
C.	oxide	528.5	212.5	1.4	2.1	4.1	7.2	1.0
D.	chloride	597.0	164.6	1.3	2.1	3.1	6.8	1.2
E.	iodide	72.8	87.7	1.1	1.6	1.4	4.9	2.0

Ratio for each tissue is avg of 15 mice in each group.

in 24 hours. Translocation from the scalp during this period was about at the level of the spread from the tail after 30 to 60 minutes.

Series C and D. About one-half of Ag^{111} oxide and Ag^{111} chloride remained in the tail and much higher proportion in the scalp within at least 24 hours. Translocation of both compounds from the tail into the liver was much lower than for Ag^{111} nitrate and lactate, and it was considerably lower from the scalp; however, storage in the spleen was higher than in any other series.

Series E. As in the case of Ag^{111} lactate only a minor share of Ag^{111} iodide was stored in the tail within 10 minutes after injection and it was slowly depleted afterwards; pickup by the liver was surprisingly low. The pattern of distribution from the scalp resembled closely that of group 5 (amputation after 3 hours) of the tail experiment.

Maxima and minima for each group varied mostly within the range of 20 to 30% of the average. In our experiments with neutral Ag^{111} phosphate, range of individual variations was much wider (due to the higher tendency of this material to clumping) and averages were not included in this report.

Discussion. Each of 5 different compounds of the same isotope showed a different pattern of dislocation from site of injection. The differences can be attributed only to variations in physicochemical properties of compounds formed by the same radioactive metal, Ag^{111}. Kyker(4) outlined very clearly a systematic approach to interpretation of various patterns in distribution of radioisotopes. He indicated that "the pH of body fluids is favorable to formation of radiocolloids when carrier free or very high specific activity material is in use"; moreover, he emphasized that "the charge, size, and chemical nature are prominent factors that influence distribution of a colloidal preparation."

Initial storage of soluble Ag^{111} compounds (Ag^{111} nitrate and lactate) at site of injection can be explained only as their fixation on tissue, tissue fluid, and blood proteins with formation of colloidal particles; their transportation in the blood and interception by liver and kidney will account for the rise of blood and liver radioactivity. For very acid Ag^{111} nitrate the combination even of low doses with blood and tissue protein resulted in a corrosive and often lethal effect. On the contrary, the weaker acid Ag^{111} lactate offered for eventual therapeutic application advantages of good tolerance by the animal and of relatively even distribution in the body.

The insoluble, coarsely particulate compounds oxide and chloride, were transported in the blood stream only slowly and in small amounts from the tail (about ⅓ remained at the site of injection after 24 hours and about 1/5 after 72 hours) and at a still lower rate (translocation of about 1/6 after 24 hours) from the scalp. The material was taken up from the blood by liver, spleen, and kidneys, and partly by reticuloendothelial elements in these organs. Previous work(5) has shown that Ag^{111} oxide was transported in connective tissue by lymphatics which became clogged by agglomeration of particles (in their lumen). This phenomenon and the presence of several large vessels in the tail may account for the higher rate of translocation from the tail. Higher tendency of Ag^{111} chloride particles to be agglutinated in tissues, perhaps through their reaction with tissue and tissue fluid, may explain their low rate of translocation as compared to Ag^{111} oxide. Both insoluble compounds are suitable, due to their ability to be deposited and stored in requisite areas, for topical treatment of malignant conditions and perhaps also of some benign pathological tissue structures(5).

Poor vascularization and large amount of connective tissue in the scalp may account for relatively slow and scanty translocation of any compound from site of injection. Vice versa, rapid and copious translocation from the tail may be correlated with its high vascularization and low content of connective tissue.

It should be noted that according to our reports on Y^{90} chloride and Ag^{111} oxide(2,5) and our unpublished results with Ag^{111} iodide the pattern of translocation for an insoluble compound given topically was considerably modified by the presence of tumor tissue in the injected area: storage of the isotope in this area was considerably higher than in normal tissues due to occlusion of lymph channels and tissue spaces by growing malignant elements (and frequently also of blood vessels by hemorrhages). This is the rationale for therapeutic trial of above compounds in tumor-bearing mice which we are carrying out at the present time.

Summary and conclusions. 1) Data were obtained in mice on translocation of 5 radioactive silver Ag^{111} compounds at various intervals after injection into the tail by a previously described method of serial tail amputations and 24 hours after injection into the scalp. 2) Ag^{111} nitrate storage rate at site of injection was higher than for another soluble compound—Ag^{111} lactate, but lower than for 2 insoluble coarsely particulate compounds—Ag^{111} oxide and chloride; on the contrary, pick-up by liver and blood was highest for soluble compounds. 3) Translocation of each compound from the scalp followed the pattern of its translocation from the tail but at a much slower rate. 4) In the above experimental conditions the fate of various Ag^{111} compounds in the body of the mouse depended mainly a) on the physico-chemical properties of the compound (solubility, pH, ability to form colloidal complexes with tissue fluid and blood, size and tendency of insoluble particles to clump) and b) on biological conditions at site of injection. 5) The planned use of this method in tumor-bearing mice is presumed to be helpful for selection of the choice compound for local or systemic therapeutic application.

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Antihypertensive Properties of Furazolidone. (24621)

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Sustained diastolic hypertension is associated with progressive decrease in renal function and increase in cardiac and cerebrovascular complications(1). Effective reduction of blood pressure arrests this progressive vascular deterioration(2). Although potent vasodepressor drugs have been developed, intolerance to certain of their actions has prolonged the search for new therapeutic agents which may obviate these difficulties(3). Differing modes of antihypertensive activity are being explored to gain insight into pathogenesis of primary hypertension, thereby affording a more rational therapeutic approach. Recent observation that blood pressure might be reduced during initial clinical pharmacologic

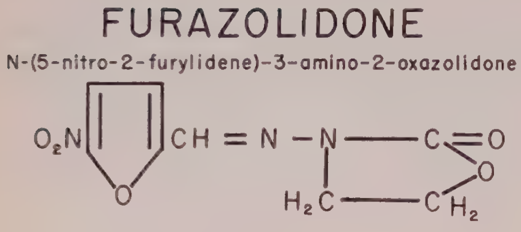

FIG. 1.

studies of furazolidone (Fig. 1) was unexpected(4). Furazolidone failed to reduce blood pressure in hypertensive rats (personal communication) and previously developed nitrofuran compounds were not found to be vasodepressor. The present study was undertaken to define any antihypertensive properties of furazolidone* and to explore its possible application and limitations in treatment of patients with hypertensive cardiovascular disease.

Materials and methods. Twenty-two ambulatory patients were studied. Patients with demonstrable secondary hypertension were excluded. Ten patients had mild, 10 moderate and one severe hypertension. In addition, one normotensive subject was included to differentiate hypotensive from antihypertensive action. The age range was 37 to 73 years with mean of 56.4 years. Fourteen patients were Negroes, of whom 9 were females and 5 were males. Of eight Caucasians, there were 5 females and 3 males. Each patient received a physical examination initially and a comprehensive history was taken. Clinical observations were repeated at weekly intervals throughout investigation. We recorded blood pressure and pulse rate in supine, sitting and standing positions. All recorded blood pressures were averaged for the control period and for period of effective drug administration (D). Initial laboratory examinations included complete blood cell count, urinalysis, blood urea nitrogen determination, x-ray of chest, electrocardiogram and stool culture. Blood cell count, urinalysis and stool culture were repeated bi-weekly. Tests of hepatic function and assays of serum electrolytes and blood urea nitrogen were repeated at intervals in certain patients. A matched placebo was administered throughout control period. Furazolidone tablets of 100 mg were then substituted in divided doses, from 300 to 1,250 mg daily. Duration of treatment with the drug was from 1 to 6 months. After additional period of placebo administration, 3 patients received a second course of furazolidone therapy.

Results. Twenty of the 22 patients studied had a reduction in mean arterial blood pressure (calculated as diastolic pressure plus one-third of pulse pressure) averaging 28 mm Hg, range 13 to 44 mm Hg (Table I). Systolic and diastolic pressures were decreased by average of 42 and 20 mm Hg respectively. An example of antihypertensive response is seen in Fig. 2. It was necessary to withdraw therapy because of side effects, in the remaining 2 patients before any antihypertensive action occurred. Two patients had an orthostatic blood pressure reduction. Several others complained of orthostatic weakness and dizziness which was not correlated with postural hypotension. The normotensive individual experienced hypotensive response of calculated mean blood pressure of 23 mm Hg.

TABLE I. Effect of Furazolidone upon Blood Pressure.

			Blood pressure*			Effective dose, mg	Onset of B.P. response, wk
Age	R	Sex	Control	D	Change		
67	W	♂	135	107	–28	800	2
56	N	♂	158	115	–43	1000	3
† 49	N	♂	127	95	–32	1000	4
† 62	N	♀	128	97	–31	800	3
65	W	♀	127	106	–21	300	4
‡ 57	N	♀	129	103	–26	600	3
† 56	N	♀	130	114	–16	800	3
44	N	♀	128	105	–23	800	4
53	N	♀	145	101	–44	800	4
‡ 54	N	♀	139	106	–33	400	5
‡ 55	N	♂	129	111	–18	600	3
49	W	♂	134	112	–22	800	5
70	W	♀	140	127	–13	800	4
45	N	♀	128	88	–40	800	1
73	N	♀	134	105	–29	800	4
†§73	W	♀	136	121	–15	800	4
‡ 52	W	♂	127	85	–42	1000	7
†§65	N	♂	123	103	–20	800	6
‖ 46	N	♂	102	79	–23	800	7
‡ 71	W	♀	126	105	–21	1000	1

* Calculated MAP.
† Side reactions necessitated stopping drug.
‡ Developed tolerance.
§ Orthostatic effect.
‖ Normotensive.

The average daily dose required for maxi-

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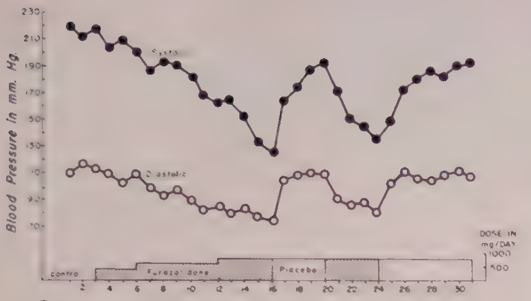
* Furnished as Furozone® by Eaton Labs.



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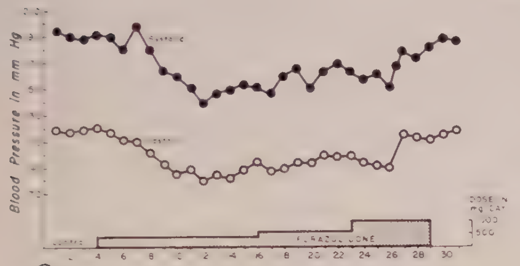

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FIG. 2. Blood pressure response to furazolidone.
FIG. 3. Development of tolerance to furazolidone.

mal effect was 775 mg with range of 300 to 1,000 mg. Attempts to reduce dosage after satisfactory effect was achieved, resulted in elevation of blood pressure. Initial antihypertensive action was manifest as early as one week, upon reaching an effective dose level; maximum response was usually attained in 3 to 4 weeks, occasionally somewhat later. Five patients became refractory to hypotensive action during our study (Fig. 3). This occurred even though dosage was increased to 1,250 mg daily. After withdrawal of drug and substitution of placebo, the blood pressure returned to its previous high levels in average of 4 weeks, with range of 1 to 8 weeks.

Furazolidone is considered to be excreted in part in the urine largely as chemically altered products. Therefore, color of urine became a characteristic amber at doses of 400 mg or more daily, intensity of color proportional to dose. This afforded an excellent verification that medication was taken as prescribed. Similarly, yellow tint of sclera developed in several patients. Since many were Negroes, the possibility of some yellow discoloration of skin could not be excluded. Laboratory studies failed to demonstrate hepatic dysfunction or hemolysis.

Body weight loss averaging 6.3 lb occurred in 12 patients, 8 of whom complained of anorexia. Nausea and vomiting were present in about one-third of patients. Other gastrointestinal effects included abdominal cramps and diarrhea in 4 and 3 patients respectively. Changes in stool flora were produced in one-third of the patients. These occurred as early as 2 weeks after initiation of therapy and included complete suppression of normal flora, appearance of Clostridia, increase in *Streptococcus viridans* and *S. hemolyticus* or replacement of normal flora by any 1 or all of aforementioned bacteria. Generalized pruritis occurred in 2 patients. Purpura, predominantly involving the 4 extremities with small confluent lesions, occurred in one patient 2 weeks after increasing the dose to 600 mg from previous level of 400 mg daily. Peripheral blood smear, platelet count and coagulation studies were normal. The purpura resolved in a few days following discontinuation of furazolidone. Anemia developed in one Negro female, improved on withdrawal of furazolidone and recurred upon reinstitution of therapy. Transitory hemic eosinophilia was observed in 2 patients, but could not be definitely related to the drug. Other infrequent complaints included a feeling of anxiety, cardiac palpitation and twitching of fingers. No abnormalities of serum electrolytes, blood urea nitrogen, urinalysis nor hepatic function were observed.

Discussion. The magnitude and consistency of blood pressure reduction achieved by furazolidone is of considerable clinical import. Both systolic and diastolic pressures were reduced, the former more than the latter, resulting in diminution of pulse pressure. The action appeared to be hypotensive rather than purely antihypertensive.

Of particular interest is the possibility that furazolidone may reduce blood pressure by a mechanism not evoked by currently available drugs. Although some evidence of orthostatic effect was observed, absence of other indications of sympathicolytic or parasympathicolytic action make ganglion blockade an unlikely principal explanation for the effect. The possible correlation of body weight loss with blood pressure reduction was considered. Of the 12 patients who lost 5 pounds or more, one-half evidenced a temporal relationship. Time curves did not coincide in the others. Upon discontinuation of the drug, weight was often regained more slowly than blood pressure was reelevated. In approximately two-thirds of the patients, change in stool flora coincided with the hypotensive effect. However, in one of these patients, a second course of furazolidone therapy failed to show such relationship. Consequently, it is not yet possible to draw conclusions regarding possible mechanism of hypotensive action.

Dosage required to lower blood pressure optimally in this study was considerably higher than that which has been recommended for enteric antibacterial therapy. At such high doses, the adverse effects, most frequently gastrointestinal in nature, definitely limit application of the drug in treatment of hypertension. Development of a refractory state to the vasodepressor action is a problem which has not been solved by increasing the dose. However, furazolidone may represent a new point of departure from which other derivatives may retain potent antihypertensive action dissociated from the aforementioned limitations.

Conclusions. Furazolidone in adequate dosage exerts a significant hypotensive action. The exact mechanism whereby this occurs remains obscure. Frequency and severity of adverse effects and development of a refractory state limit application of the drug in treatment of patients with hypertension. It is to be hoped that derivatives may dissociate the hypotensive from the unwanted effects.

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Effect of Antihistaminic and Antiserotonin Drugs on Vascular Responses to *E. coli* Endotoxin in the Cat.* (24622)

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Devices by which endotoxin causes shock and death are only partly understood. The resemblance of its early vascular effects in certain species(1) to changes seen in anaphylaxis (2) has suggested that there may be certain mechanisms common to both situations. Since histamine and 5-HT (5-hydroxytryptamine) are known to be released in anaphylaxis(3) it seemed possible that they may also be set free in reactions to endotoxin and that antihistaminic and antiserotonin drugs might therefore block some effects of endotoxin. Chlorpromazine, which has both antihistaminic and adrenergic blocking activity(4) has been shown to protect mice against lethal effects of endotoxin(5). Dibenzyline has also been found to have a protective effect(6,7). In the present study, a combination of dibenamine and pyrilamine blocked the *immediate* systemic hypotensive effect of small doses of endotoxin in cats.

Methods. In the cat, endotoxin causes a sharp rise of PAP (Pulmonary artery pressure) and of total pulmonary vascular resistance. This is followed by a decline in BP (systemic arterial blood pressure) from which there is usually a temporary recovery(8). Cats were anesthetized with intraperitoneally injected sodium pentobarbital (35 mg/kg). After cannulation of the trachea and carotid artery, the chest was opened to permit insertion of a polyethylene catheter into the pulmonary artery. The PAP and BP were meas-

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TABLE Ia. Responses to Histamine (20 μg/kg) before and after an Antihistaminic.

Antihistaminic and dose	BP change		PAP change	
	mm Hg			
	Before	After	Before	After
Tripellenamine, 2.5 mg/kg, and diphenhydramine, 2.5 mg/kg	–65	–35	– 2	
Chloroprophenpyridamine maleate, 2 mg/kg	–22	–18	+ 3	– 1
Promethazine				
25 mg/kg	–40	–20	+ 8	+5
20 "	–33	–31	+ 1	+1
Pyrilamine, 5 mg/kg	–51	– 7	+16	+1
Idem	–60	–17	+12	– 2
"	–33	–34	+13	– 2
"	–85	–40	+12	+2
"	– 5	–30	+15	+3

ured and recorded with Statham strain gauges and a Sanborn 2-channel recorder. The purified endotoxin from *E. coli* was prepared by a modified Boivin technic as described by Spink and Anderson(9). 5 mg/kg of this preparation was fatal to 7 of 8 cats when given intravenously.‡ A different cat was used for each test with endotoxin. All injections were made through a polyethylene catheter which had been inserted in the external jugular vein. Dilutions of histamine and 5-HT in 5% dextrose solution were made afresh each day. All doses are stated in terms of the base. Dibenamine from a stock solution in acidified propylene glycol and ethanol was diluted with normal saline immediately prior to injection. LSD (lysergic acid diethylamide) was injected as supplied,§ as was the commercially obtained pyrilamine. To allow time for the blocking drugs to become effective, injection of endotoxin was delayed for an hour after their administration.

Results. The effect of several antihistaminic drugs on BP and PAP responses to a standard dose of histamine is shown in Table Ia. The effects of dibenamine and LSD on pressure responses to 5-HT are shown in Table Ib. The blocking action of these agents against histamine and 5-HT was unpredictable and incomplete. It was therefore decided to use only 0.05 mg/kg of endotoxin, a dose 1/100 that used in dog studies reported from this laboratory. This amount of endotoxin was sufficient to consistently cause a clearly observable effect.

The results for both control and pretreated cats are shown in Table II. The most clear-cut protection was seen from a combination of dibenamine and pyrilamine. The BP was not significantly affected by endotoxin in any of 5 cats so pretreated. Fig. 1 contrasts the record obtained from such an experiment to that obtained from a control animal. The findings in the 15 control cats make it unlikely that the lack of a blood pressure fall in the pretreated animals was related to their lower initial pressure. The initial BP in the control cats ranged from 75 to 155. In these 2 animals BP fell 45 and 0 mm Hg respectively. The correlation coefficient between the initial BP of the control cats and the fall in pressure after endotoxin was only +.128 ($t = .465$, $P > .50$).

Dibenamine alone was effective in all but one of 5 trials and its average effectiveness was significant at the .05 level. Pyrilamine alone had no significant protective action. Neither LSD by itself nor LSD with pyrilamine gave significant protection in groups of 4 cats. When data for these 2 groups were pooled however, degree of protection against acute hypotensive effects of endotoxin became statistically significant at the .02 level. The effect of a large dose of endotoxin (5 mg/kg) was unaltered by any of the agents or combinations shown. The effect of these various

TABLE Ib. Responses to 5-HT (20 μg/kg) before and after Blocking Agent.

Blocking drug and dose	BP change		PAP change	
	mm Hg			
	Before	After	Before	After
Dibenamine, 15 mg/kg	–38	–15	+ 6	+1
Idem	–15	–13	+27	0
"	–45	–20	+ 2	+1
LSD, .5 mg/kg	–25	–20	+ 6	+5
Idem	–35	–20	+ 6	– 2
"	–25	–13	+ 9	+3
"	30	–35	+ 4	– 5

---

‡ Endotoxin was made available through the courtesy of Dr. Wesley W. Spink, Univ. of Minnesota.

§ LSD was provided in ampoules containing 0.1 mg/ml of LSD through courtesy of Dr. R. Bircher of Sandoz Pharmaceuticals.

TABLE II. Responses to Endotoxin with and without Blocking Drugs.

No. cats	Drug before endotoxin	BP mm Hg	BP change mm Hg	% change BP	P	PAP mm Hg	PAP change mm Hg	% change PAP	P
15	None (control)	112	−50	−45.0 ± 9.0*		16.6	+12	+73 ±17 *	
5	Dibenamine, 15 mg/kg, and pyrilamine, 5 mg/kg	78	+ 2	+ 2.8 ± 3.1	<.01	11.2	+ 3	+26.8 ±10.3	>.10
5	Dibenamine, 15 mg/kg	66	9	−14.8 ± 8.5	<.05	14.2	+ 6	+49 ±34.2	>.10
5	Pyrilamine, 5 "	97	−21	−17.6 ±15	>.10	17.4	+ 7	+52 ±23.5	>.10
4	LSD alone, .5 "	65	− 6	− 8.0 ± 5.1	>.05	20.0	+ 9.7	+48.7 ±15.8	>.10
4	LSD, .5 mg/kg, and pyrilamine, 5 mg/kg	68	− 8	−11.6 ± 2.3	>.05	19.0	+ 2.5	+15.4 ± 6.9	>.05
8	LSD, .5 mg/kg, alone or plus pyrilamine, 5 mg/kg	67	− 7	− 9.9 ± 3.21	<.02	20.0	+ 6.1	+32.0 ±10.4	>.05

* ± S.E.

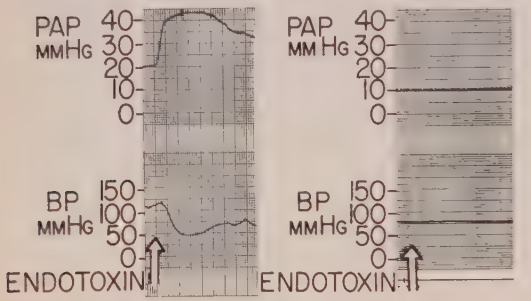

FIG. 1. Left side of illustration shows effects of endotoxin (.05 mg/kg) on pulmonary artery pressure (PAP) and arterial pressure (BP). Right side shows lack of effect of endotoxin in a cat which had received pyrilamine (5 mg/kg) and dibenamine (15 mg/kg). Heavy time lines are 2.5 sec. apart.

drugs on PAP responses to endotoxin was not statistically significant.

Tetraethylammoniumchloride (5 mg/kg) was ineffective in 3 cats. BAS (benzyl analogue of serotinin, 1 mg/kg)‖ was administered by stomach tube to 2 cats for 3 successive days. The effect of endotoxin given on the third day was unaltered.

Discussion. These data indicate that prior administration of antihistaminic and antiserotonin drugs can prevent acute hypotensive effects of small doses on endotoxin in cats. It is possible that in a larger number of cats partial protection could be shown for pyrilamine or LSD alone. Tetraethylammoniumchloride and BAS had no effect on the acute response to endotoxin in 3 and 2 trials respectively.

Previous workers have shown that dibenzyline and chlorpromazine improve *survival* rate of mice given endotoxin(5,6). Similar results have been shown for other forms of shock (10). Boquet and Izard found that dibenamine greatly diminished vasoconstriction produced by endotoxin in the rabbit's ear(11). Lillehei and MacLean(7) have recently shown that either dibenzyline or chlorpromazine greatly improve survival rate in dogs given endotoxin. Initial BP response was not affected. Our data concern the initial *acute* circulatory response in cats. The results are compatible with the thesis that histamine and 5-HT are released in the initial stages of the endotoxin reaction. Although there was no blocking of the response to *large* doses of endotoxin, neither was there blocking of the responses to large doses of histamine or 5-HT. It is, of course, quite possible that the effect was on other vasoactive amines or was a nonspecific action against totally different agents. It is improbable that epinephrine blockade alone is a factor, since the early endotoxin response is so different from that seen after epinephrine.

Despite the statistically insignificant effect of these drugs on the PAP response to endotoxin, it is likely that the pulmonary vascular constriction was sufficiently abated that the left ventricular output and hence the BP were not significantly affected by the endotoxin.

Summary. Pretreatment with dibenamine and pyrilamine, dibenamine alone, or LSD with or without pyrilamine was found to diminish or prevent the acute hypotensive response of the cat to small doses of endotoxin.

‖ BAS and instructions for its administration were kindly supplied by Dr. D. W. Woolley, Rockefeller Inst.

It is a pleasure to acknowledge the encouragement and guidance of Dr. Maurice B. Visscher.

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Detection and Isolation of Formiminoglutamic Acid from Urine in Folic Acid Deficiency in Humans.[*][†] (24623)

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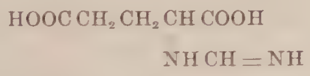
Silverman and coworkers(1) found that rats on folic acid (FA) deficient diet excreted in the urine a compound which was converted to glutamic acid when heated or exposed to alkali. Excretion of the glutamate precursor was associated with severity of FA deficiency (1) and increased by supplementing the diet with histidine(2). Subsequent chemical evidence(3) indicated that the glutamate precursor was N-formiminoglutamic acid (formamidinoglutaric acid):



$$\mathrm{HOOC\,CH_2\,CH_2\,\underset{NH\,CH=NH}{CH}\,COOH}$$

Formiminoglutamic acid (FIGLU)

That a metabolic relationship between FA and FIGLU exists has become clearer with recent enzyme studies. Thus it has been shown that FIGLU may arise from degradation of histidine by liver homogenates and *Pseudomonas* extracts(4) and that tetrahydrofolic acid (THFA) functions in certain mammalian enzyme systems(5,6) as acceptor of formimino group of FIGLU giving formimino-THFA(6) and glutamic acid. Our study was undertaken to determine if FIGLU might be excreted in FA deficiency in man. Such findings would support concept that FA functions in formimino group transfer, and suggest detection of FIGLU in human urine be considered as a diagnostic tool for folic acid deficiency in man. Preliminary findings have previously been reported by us(7,8,9).

Methods. Twelve children with acute leukemia receiving various types of chemotherapy, one woman with macrocytic anemia of pregnancy and 5 normal subjects were studied. Highlights of clinical findings in addition to data in Table I, are: Subj. 1: A.B. Acute myeloblastic leukemia associated with mongolism, no previous antileukemic therapy, in clinical and hematologic relapse, blood WBC 28,600 mm^3, when urine collected; Subj. 2: S.M. Subacute lymphatic leukemia, no response to previous 6-mercaptopurine (Purinethal)® or steroid therapy, in clinical and hematological

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relapse, blood WBC 15,600 mm³, when urine collected; Subj. 3: R.F. Acute myeloblastic leukemia, excellent response to previous 6-mercaptopurine (Purinethal)® therapy followed by complete relapse; 6-MP discontinued 3 weeks previously, aminopterin 0.5 to 1.0 daily dose; moderate stomatitis, blood WBC 1,500 mm³, Subj. 4: D.A. Acute stem cell leukemia in clinical and hematologic remission with amethopterin (Methotrexate)® administered intermittently as maintenance therapy, received 105 mg prior to urine sample "d"; blood and marrow "practically" normal. Urine sample "e" collected 3 weeks later after amethopterin (Methotrexate)® discontinued because of stomatitis. *Urine for FIGLU isolation and identification* was collected from this patient in large quantities for several days after amethopterin resumed at average daily dose of 3.75 mg for several weeks, with patient still in clinical and hematologic remission. Subj. 5: M.R. Acute lymphoblastic leukemia, in clinical and hematologic remission, amethopterin 2.5 mg daily as maintenance therapy. Subj. 6: S.F. Acute stem cell leukemia, going into relapse despite continuance of amethopterin, 2.5 mg daily, following excellent clinical and hematologic remission. Subj. 7: W.M. Acute myeloblastic leukemia, no clinical or hematologic response despite 5 mg daily dose amethopterin when sample "h" collected; amethopterin discontinued because of stomatitis and leukopenia, blood WBC 1,600 mm³, when sample "i" collected. Subj. 8: J.P. Acute granulocytic leukemia, no clinical or hematologic remission although amethopterin administered 2.5 mg daily in first 8 weeks, 3.75 mg daily next 2 weeks and 5 mg last week. No mouth toxicity, blood WBC 2,000 mm³, marrow 93% "blasts" Subj. 9: M.S. Acute stem cell leukemia, in complete clinical and hematologic remission following 6-mercaptopurine (Purinethal)® regimen at 100 mg daily. Subj. 10: T. K. Acute micromyeloblastic leukemia, in clinical and hematologic relapse on 6-mercaptopurine regime of 37.5 mg daily. Subj. 11: T.B. Acute stem cell leukemia, in complete clinical and hematologic remission on 6-mercaptopurine regimen of 42.5 mg daily. Subj. 12: L.B. Acute stem cell leukemia, in partial hematologic remission on prednisone (Metacorten)® 60 mg daily. *Macrocytic anemia of pregnancy.* Subj. 13: M.A. discovered on first postpartum day, characterized by typical findings of peripheral blood macrocytosis, anemia (hematocrit 19%), leukopenia (WBC 3,000 mm³) and hypersegmented polymorphonuclear leukocytes. Bone marrow aspirate showed erythroid hyperplasia with typical megaloblastic features. On third day after diagnosis established, folic acid, 20 mg daily was administered orally for next 16 days. Patient's blood showed characteristic reticulocyte response reaching peaks of 13.5% on fifth and eighth day after start of folic therapy. When urine sample "p" collected, hematocrit had risen to 37.5%, WBC to 7.500 mm³. Bone marrow was normoblastic. *Control subjects* 14 to 18 incl. were normal individuals on average diets and showed no hematologic abnormalities. Several patients with iron deficiency anemia and Addisonian pernicious anemia were also studied as control subjects. Twenty-four hour *urine specimens* were collected under toluene in bottles containing sufficient glacial acetic acid to give final concentration of 1% and were held at 4°C, until prepared for assay. All urines were assayed within 7-14 days after collection. Upon arrival at laboratory, urine was adjusted to pH 6.5-7, clarified by filtration, and an aliquot sterilized by Seitz filtration. A portion of sterile filtrate was autoclaved 15 minutes. Urine specimens from individuals treated with aminopterin (4-amino-FA) or amethopterin (Methotrexate,® 4-amino - 10 - methyl - FA) were clarified by filtration and then stirred with 2% charcoal (Darco G-60) at pH 2-3 for 30 minutes, filtered and then sterilized as described above. The charcoal absorption served to remove FA antagonists which could interfere with the microbiological assay. *Microbiological assay* for glutamic acid using *Lactobacillus arabinosus 17-5* was employed following procedure of Henderson and Snell (10) and Hac *et al.*(11). Aliquots of *unheated* sterile urine and autoclaved (or *heated*) sterile urine were added aseptically to a series of 11 x 180 mm test tubes containing 1 ml of sterile, double strength, glutamic acid free medium(10) and the volume of

TABLE I. Microbiologic Evidence for Urinary Excretion of Formiminoglutamic Acid.

Clinical status						Urinary glutamate activity		
Subject	Age (yr)	Sex	Disease state	Therapy and duration	Sample No. and date	Unheated aliquot	Heated aliquot	Ratio H/U
				Acute leukemia				
A.B.	2½	♂	Relapse	None	a. (2- 3-55)	49	14	.3
S.M.	10	♂	"	"	b. (4- 4)	140	50	.4
R.F.	8	♂	Partial remission	Aminopterin 11 days	c. (7-12)	78	109	1.4
D.A.	14	♀	Remission	Methotrexate* 2 wk	d. (6-14-56)	85	99	1.2
				After discont. 2 days	e. (7- 6)	35	10	.3
M.R.	5	♀	"	Methotrexate 4 wk	f. (7-17)	23	126	5.5
S.F.	3	♀	Relapse	Methotrexate 6 wk	g. (6-28)	140	304	2.2
W.M.	10	♂	"	Methotrexate 6 wk	h. (6- 5)	63	75	1.2
				After discont. 3 days	i. (6-11)	41	15	.4
J.P.	5½	♂	"	Methotrexate 8½ wk	j. (6-21)	74	144	1.9
M.S.	9	♂	Remission	Purinethal† 6 wk	k. (11- 7)	50	20	.3
T.K.	2½	♂	Relapse	Purinethal 2 wk	l. (")	51	<10	.2
T.B.	4	♀	Remission	Purinethal 13 wk	m. (")	64	41	.6
L.B.	10	♀	Partial remission	Metacorten‡ 3 wk	n. (11-12)	60	<10	.2
				Macrocytic anemia of pregnancy				
M.A.	22	♀	Post-partum 1 day	None	o. (12- 3-55)	77	99	1.3
			Post-partum 6 wk	Folic acid 16 days	p. (1-16-56)	98	19	.2
				Controls				
AHB	6	♂	Normal child		q. (7-12)	75	<10	.1
SAB	12	♂	*Idem*		r. (")	63	"	.2
HPB	Adult	♂	"		s. (")	125	"	.1
LAH	"	♂	"	—	t. (7-14)	99	"	.1
AMA	"	♀	"		u. (")	57	"	.2

* Amethopterin. † 6-mercaptopurine. ‡ Prednisone.

tubes was adjusted to 2 ml. The tubes were then inoculated with a "heavy inoculum"(11), and the assay incubated for 72 hours at 37°C. Growth was estimated turbidimetrically.

Presence of FIGLU in test sample was inferred from these considerations: Glutamic acid is fully active for *L. arabinosus* whether it is heated (autoclaving 15 minutes at pH 6.5-7) or added aseptically. Glutamine, on the other hand, is destroyed by this heating procedure and is active only if added aseptically. FIGLU cannot be utilized by *L. arabinosus* as a source of glutamic acid(12) but when it is autoclaved under conditions described above, it is hydrolyzed almost completely to glutamic acid (Luhby, A. L. and Cooperman, J. M.—Unpublished data). Growth response to *unheated* urine samples was regarded as a response primarily to glutamine for free glutamic acid content of normal urine is low relative to glutamine(13). Thus any significant growth response to *heated* urine samples was regarded as presumptive evidence for presence of FIGLU.

Results. Table I gives the glutamic acid excretion patterns of the subjects. Urine from normal adults and children (subjects 14-18) contained ample glutamic acid activity when urine samples were not heated, but were very low in activity when heated. This ratio (H/U) is low in normal individuals. Urine specimens from children with acute leukemia, either untreated (subjects 1 and 2), or who were being treated with Purinethal® (subjects 9-11) or Metacorten® (subject 12) had H/U values similar to those of normal children. However, urine obtained from 6 children with acute leukemia who were on maintenance therapy with FA antagonists (subjects 3-8) increased in apparent glutamic acid content when heated (H/U ratio >1). Moreover in 2 instances (samples e and i) when the antagonist was withdrawn there was a concomitant decrease in the H/U ratio. Thus an increased H/U ratio is associated with FA antagonist therapy, but not with leukemia. Following administration of folic acid and complete hematologic and clinical remission, the H/U ratio (sample p) decreased to 0.2. A limited number of urine specimens from individuals with iron deficiency or Addisonian pernicious anemia have not shown evidence of FIGLU.

Isolation of glutamate precursor from urine. 3.1 liters of urine were collected from a 13-year-old child with acute leukemia in remission, Subject 4, while on maintenance therapy with amethopterin (*cf.* Experimental). Urine was clarified by filtration, adjusted to pH 2.5 with HCl, and stirred for 30 minutes with 31 g Darco G-60 activated charcoal. The charcoal was removed by filtration on Buechner funnel with the aid of Filtercell. An aliquot of the clear filtrate adjusted to pH 6.5 and autoclaved 15 minutes contained 180 μg/ml glutamic acid activity by microbiological assay. The charcoal filtrate was reduced to 1 liter by vacuum distillation at 25°C and the glutamate precursor isolated by ion exchange chromatography following the procedure of Silverman *et al.*(1). The concentrate was passed through a Dowex 50 (200 x 400 mesh, crosslink 8) H+ column (1½″ wide packed to depth of 20″) at 100 ml/hr., and 1 liter of water and 0.5 liter quantities of 1.5 μg and 10% acetic acid solutions passed successively through the column. The column was then placed on a fraction collector and developed with 1% sulfuric acid (flow rate, 50 ml/hr). Aliquots of all fractions were spotted on paper and sprayed with ninhydrin, or first exposed to ammonia vapor for 1 hour, aerated, then sprayed with ninhydrin. All fractions that were ninhydrin negative before ammonia treatment, but gave a color indicative of an α-amino acid after ammonia exposure, were pooled. 250 ml of such a pooled fraction was placed in a precipitating dish in icebath and 9 ml of 25% mercuric acetate in 0.2 M acetic acid was slowly added to form a mercury salt (12a). The precipitated mercury salt was collected at the centrifuge and washed successively with water, ethanol, and ether, air dried, to give 420 mg of nearly white material. An attempt was made to further purify this mercury salt by reprecipitation with mercuric acetate, with the glutamate derivative in excess to minimize contamination with mercuric oxide: 350 mg of mercury salt was suspended in 30 ml 0.01N sulfuric acid. Hydrogen sulfide was passed through it until precipitation of mercuric sulfide was complete. The latter was removed by centrifugation, and the clear filtrate vigorously aerated. Aliquots of this filtrate when chromatographed on paper had a major component indistinguishable in 2 solvent systems from synthetic FIGLU (Fig. 1). The remainder of the filtrate was treated with 1.3 ml mercuric acetate reagent as described above to finally give 165 mg of white mercury salt. Elemental analysis of this derivative was as follows:

$C_6H_{10}N_2O_6Hg_2$ (606)

Calculated: C, 11.9; H, 1.7; N, 4.6
Found: C, 10.5; H, 1.7; N, 4.3

Degradation products of isolated mercury salt. The mercury salt was subjected to various hydrolytic procedures shown in Table II. A mole of ammonia N was released/mole of mercury salt by either acid or alkaline hydrolysis. 0.7 mole of glutamic acid/mole of product was recovered by acid hydrolysis. When a cruder mercury salt was either autoclaved or let stand at pH 12 for 24 hours at 25°C, appearance of glutamic acid was demonstrated by paper chromatography(12). The chemical and microbiological properties de-

TABLE II. Products of Hydrolysis of Isolated Mercury Salt.

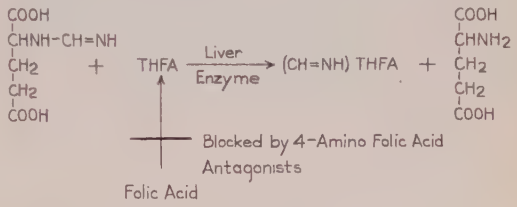
Method of hydrolysis*	Hg salt used, μM	Ammonia N,† μM	L-glutamic acid,‡ μM	Recovery for $C_6H_{10}N_2O_6Hg_2$, %
1 ml 4.5 N KOH, 120°C, 1.5 hr	20	18.6		93
1 ml 6 N HCl, " 15 "	"	19.3		97
Idem	"		14	70

* Hg salt suspended in indicated reagents in 1 ml ampul, the ampul sealed and autoclaved.
† Determined by Nesslerization.
‡ Hg^{++} removed from hydrolysate by precipitation as sulfide, and glutamic acid content of filtrate determined by microbiological assay(10).

scribed for the glutamic acid precursor isolated, indicate that it is a highly purified mercury salt of N-formiminoglutamic acid.

Discussion. It is now clear that in suitably purified mammalian enzyme systems(5,6), THFA is required for catabolism of FIGLU to glutamic acid.

One action of the 4-amino folic acid antagonists is to block one stage of the reduction of folic acid to THFA(14) as indicated in the diagram. Hence the excretion of FIGLU in children receiving folic acid antagonists very likely occurs as a consequence of insufficient THFA essential for disposition of FIGLU. This substance might also accumulate in urine if the diet were deficient in folic acid or if pregnancy reduced maternal stores of folic acid. It is thus possible that determination of FIGLU in urine may be an aid in diagnosing nutritional folic acid deficiency.



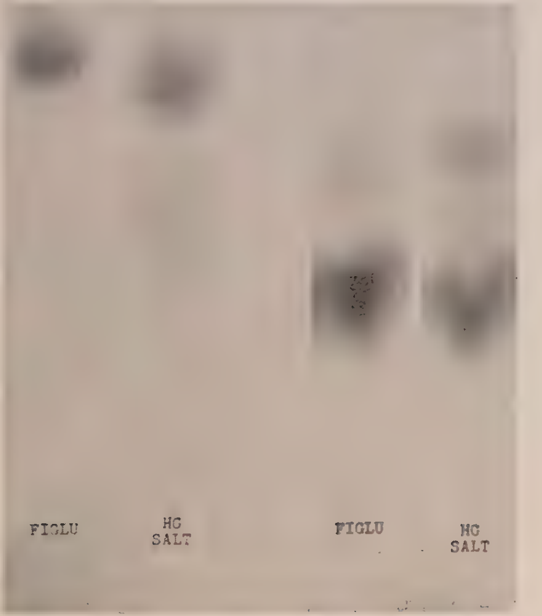


FIG. 1. Paper chromatography of synthetic formiminoglutamic acid (FIGLU) and glutamate precursor (mercury salt) isolated from urine. Solvent system first 2 chromatograms: collidine (1), lutidine (1), trimethylamine (1%), and water (1). Solvent system in last 2 chromatograms: n-butanol (40), acetic acid (10), water (50), used butanol layer. 50 γ synthetic FIGLU and .02 ml free acid of mercury salt (see text) were spotted at origin of 25 mm width Whatmann #1 paper strips, allowed to develop by ascending technic at 25°C for 40 hr, air dried, exposed to ammonia vapor 1 hr, aerated 1 hr, then sprayed with ninhydrin.

Tabor and Wyngarden recently published an enzymatic method for determination of FIGLU in urine(15). With this method Hiatt *et al.*(16) also found that FIGLU is excreted in substantial quantities in children treated with amethopterin.

FIGLU, isolated as described above from human urine, was as active as an authentic sample of FIGLU kindly supplied by Dr. Herbert Tabor, when the natural and synthetic materials were compared in the enzymatic assay(15).

Summary. 1. A microbiological assay procedure giving presumptive evidence for presence of formiminoglutamic acid (FIGLU) was carried out on urine specimens from normal individuals, children with acute leukemia receiving various types of chemotherapy, and a patient with macrocytic anemia of pregnancy. The data indicated strongly that FIGLU was being excreted in children with acute leukemia receiving 4-aminofolic acid antagonists and in macrocytic anemia of pregnancy. 2. A highly purified mercury salt of a glutamic acid precursor was isolated from urine of patient with leukemia during amethopterin therapy, and on the basis of its chemical and microbiological properties, concluded to be FIGLU. 3. It is postulated that folic acid antagonists induced a folic acid deficiency, resulting in insufficient tetrahydrofolic acid needed for catabolism of FIGLU. Consequently FIGLU accumulated. It is suggested that determination of FIGLU in human urine may be a useful aid in diagnosing folic acid deficiency in man.

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Effect of Food Deprivation on Thermal Behavior of the Rat. (24624)

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Ordinarily the laboratory rat has little or no control over his environmental temperature. When cold stressed, this organism, unlike man, does not have access to external heat control devices. Recently McCleary *et al.* (unpublished) investigated behavioral results of providing the rat with some control over environmental temperature. Rats were exposed to low ambient temperature and given a response pedal, which when depressed, activated a 10 second burst of heat from an infra red heat lamp. Under these conditions the rate of bar pressing was shown to be a function of ambient temperature. Weiss(1) has extended this work to show that the bar press rate under above conditions is also a function of restricted food intake and pantothenic acid deprivation. The present study is a further extension of the above work, using a modified dependent variable with 3 levels of food deprivation.

Method. Six adult male Sprague Dawley rats were used. The experimental apparatus was a rectangular box of galvanized steel, 15 by 24 by 10 inches. A ⅜ inch round response bar protruded into the box 4¼ inches and was located 4½ inches in front of 2 GE 250 watt infra red heat lamps with built-in red filters. The lamps were separated from the bar compartment by a partition of ¼ inch hardware cloth. Depression of the bar closed a microswitch and activated the heat lamps. At the same time a cumulative timer and response counter were energized. This apparatus was placed in a conventional refrigerator with ambient temperature at 4° ± 1°C at beginning of experimental session. Auxiliary lighting was supplied by 2 NE 40 GE glow lamps at top of side opposite the heat lamps. Preliminary training of animals consisted of exposure to experimental conditions for 2 hours before first day of recording. The animal's coat was clipped and it was placed in the apparatus and left to discover the response bar and its func-

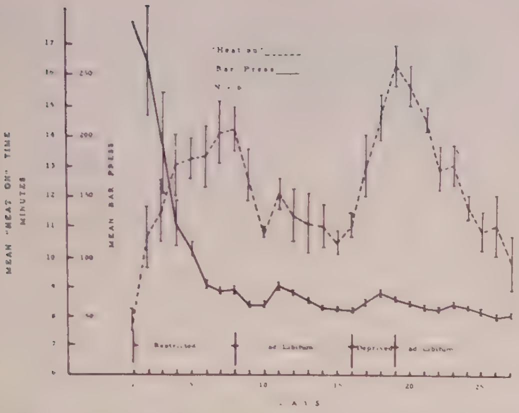

FIG. 1. Mean heat on time and bar press rate (with S.E. of means plotted) as a function of various feeding schedules.

tion. Thereafter each animal was run one hour a day for 27 consecutive days. From day one through day 8 the rats were placed in the apparatus approximately 22 hours after feeding 1½ hours on powdered chow. Days 9 through 16, the rats were placed on an *ad lib.* diet of Purina Chow pellets. After one hour session on 16th day, food was removed from the cages. The following 3 days the animals were run 24, 48, and 72 hours deprived of food. After the 72 hour run the animals were again placed on the *ad lib.* diet. Two response measures were recorded—total time that heat lamps were on, and number of bar presses.

Results. Fig. 1 summarizes the results. A sharp decline of bar press rate and a rise of heat were noted during the first 5 days. These are taken as evidence that the rats quickly learned first to press the bar and then to hold it down for longer periods of time. Further evidence for learning to hold the bar down was shown during period of starvation, when the mean heat on time was increased by 48% while the mean number of bar presses increased by only 17%. Each subject showed increase in heat on time over the 3 day period. The range of these increases from day 16, the last day of *ad lib.* feeding, to the 72 hour deprivation period was 3.2-7.5 minutes. It is on this point that the present response measure appears to present an advantage over that of bar press rate alone. Bar press rates, under conditions of predetermined 10 second heat bursts are likely to be a function, not only of heat requirements, but also of high activity level during food deprivation. In addition, high activity level would result in increased internal heat production which in turn might attenuate external needs. The present data suggest that by using heat on time as the dependent variable, a more valid picture is obtained of heat requirement.

The apparent rise in heat drive during the starvation period, as reflected in increased heat on time, probably results from the animal's deficiency of energy supply. This deficiency logically leads the rat to attempt to conserve body heat or to reduce heat loss, since body temperature is the result of a balance between heat production and heat loss.

It is pertinent to discuss the nature of reinforcement in this work. Since a bar press is not only followed by heat but also by a change in illumination it might be argued that for some reason the rat on the deprivation schedule is being reinforced by this illumination change. Davis(2) has shown that if the onset of dim illumination is made contingent upon bar pressing, the response rate rises as a function of hours of food deprivation. He believed the onset of illumination to be the reinforcing condition. In the present heat reinforcement study, however, it has been shown that the bar press rate changes very little with increasing hours of food deprivation. It is unlikely, therefore, that onset of illumination (and not the heat) is the reinforcement. The remaining possibility, that deprived rats for some reason prefer longer periods of illumination, seems equally unlikely, but future studies will utilize a filter between the lamps and the rat compartment.

A further qualification of the conclusions is needed because of the nearness of the response bar to the heat lamp. It is conceivable that under food deprivation conditions, resulting in prolonged bar depression, the heat becomes noxious. This would tend to attenuate the heat on time. Studies have been initiated to investigate this variable by placing the response bar at various intervals from the heat lamp.

Summary. Rats exposed to low ambient temperatures were trained to depress a response bar to obtain heat. The heat remained on as long as the bar was depressed. Total time of bar depression was shown to be a function of hours of food deprivation.

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Immunization of Mice with Polysaccharides of *Histoplasma capsulatum*.* (24625)

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There are significant reports on enhancement of resistance to bacterial infection following immunization with specific bacterial polysaccharides(1-4). However, little or no data are available concerning the immunizing effect of fungal polysaccharides. Salvin(5,6, 7) presented evidence of increased resistance following intraperitoneal injection of acetone-dried yeast phase *Histoplasma capsulatum*. Mice that had been immunized had fewer organisms in their tissues (spleen, liver, kidney) than nonimmunized mice. The difference between the 2 groups was most noticeable during the first 3 weeks following challenge. In nonimmunized mice, rapid growth of the fungus occurred during the first few hours after challenge; a gradual decrease in numbers of organisms was observed during the following months [Salvin(8), Grayston and Salvin(9)]. Rowley and Huber(10) showed the same sequence of events in mice that had been superinfected; however, they were unable to demonstrate any increase in resistance following immunization with a vaccine prepared from yeast phase *H. capsulatum*. Salvin and Ribi (11) found that the cell wall contained the antigenic fraction(s) responsible for the protection observed against *H. capsulatum*.

The present investigation was conducted to determine the immunogenic capacity of polysaccharide fractions of *H. capsulatum* when compared to immunizing activity of killed whole yeast phase vaccine of the same organisms.

Methods. Our previous studies on *H. capsulatum* strain G17(M) have shown this culture to have a LD_{50} for mice of 1.2 x 10^6 (95% confidence limits of 0.8-1.8 x 10^6) yeast phase organisms when injected intravenously. This culture is significantly more virulent than other stock strains of *H. capsulatum* maintained in this laboratory, and was used throughout. The method for extraction of *H. capsulatum* polysaccharides has been reported (12). The polysaccharide was prepared for use as skin test antigen. *H. capsulatum* G17 (M) broth polysaccharide was injected intraperitoneally into 60 albino mice at 4 day intervals according to following schedule: On days 1, 5, 9 and 13, the following amounts of *H. capsulatum* polysaccharide were given, mg .005, .01, .1, and 1. A second group of 60 mice was injected intraperitoneally with 1 mg of *H. capsulatum* G17(M) broth polysaccharide when fourth injection was given to the first group of mice. Mice of a third group of 60 were immunized by 2 weekly intraperitoneal injections of 1 ml of *H. capsulatum* G17 (M) formalin-killed whole yeast cell antigen (about 10^7 organisms/ml). A fourth group of 60 mice served as nonimmunized control. Ten days after last injection the 4 groups of mice were challenged by intravenous injection of varying numbers of viable *H. capsulatum* G17(M) yeast phase cells. These organisms were grown on antibiotic containing blood agar slants. The yeast phase cells were removed from the blood agar by suspension in brain heart infusion broth. Number of cells in the suspension was estimated from hemacytometer cell counts. The immunized and

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TABLE I. Mortality Ratios of Immunized and Nonimmunized Mice 21 Days after Challenge with *H. capsulatum* Strain G17(M).

Immunizing antigen	Challenge dose 1×10^6	 2.5×10^6	 5×10^7
Whole cell	1/16	2/16	9/16
Polysaccharide—4 inj.	5/18	2/17	13/18
1 "	3/12	2/12	8/15
Control (nonimmunized)	11/19	12/20	20/20

control groups of mice were each divided into 3 subgroups of 20 and challenged with 1 x 10^6, 2.5 x 10^6 and 5.0 x 10^6 yeast phase organisms. Deaths were recorded on day they occurred; however, the results were analyzed on the basis of cumulative mortality ratios after 21 days.

Results. Animal losses during immunization and within 24 hours after intravenous challenge reduced some subgroups of 20 mice to smaller numbers. The data presented in Table I suggest that some degree of protection was induced in the immunized groups. The significance of the magnitudes of differences among these data can be more clearly estimated by statistical analysis; therefore, the data were analyzed by the method of Wilcoxon and Litchfield(13).

Table II presents the calculated LD_{50} values and 95% confidence limits for each group. The results indicate that there is significant protection of mice immunized with either whole cell or polysaccharide when compared to nonimmunized mice following challenge. The LD_{50} values for mice receiving 4 immunizing injections of polysaccharide or a single dose of the same antigen were close and no significant difference could be demonstrated by the analytical method used. The relative efficacy of immunizing procedures, determined by comparison of the control group with immunized groups, was further investigated by calculation of potency ratios: these values are shown at the bottom of Table II.

TABLE II. Analysis of Mortality Ratios of Mice Immunized with Whole Cell or Polysaccharide Antigen.

Method of immunization	LD_{50}	95% confidence limits
Whole cell	8.0×10^6	4.1–15.6 $\times 10^6$
Polysaccharide—4 inj.	4.5 "	3.0– 6.75 "
1 "	4.5	2.5– 9.0 "
Control	1.05 "	.6– 1.83 "

$$LD_{50} \text{ potency ratios} = \frac{LD_{50_1}}{LD_{50_2}}.$$

Whole cell and control = 7.62 (3.18–18.2).
Polysaccharide and control = 4.28 (1.76–10.4).

The 4 injection and 1 injection polysaccharide immunized mice reacted equally to the challenge doses. Animals receiving polysaccharide were able to withstand 1.76 to 10.4 times the number of organisms which killed 50% of the controls; between 3.18 and 18.2 times more organisms were required to produce this mortality in whole cell immunized mice as in control mice. It is apparent therefore that the skin test polysaccharide preparation was able to induce a significant degree of protection in mice. One immunizing injection of 1 mg of polysaccharide material gave protection equal to that obtained by 4 injections (total of 1.115 mg) with this polysaccharide. The protection was significant but not as extensive as that induced by the whole cell vaccine.

Summary. *H. capsulatum* strain G17(M) polysaccharide was capable of producing active resistance against intravenously induced histoplasmosis in mice. However, mice immunized with a *H. capsulatum* whole yeast cell antigen showed greater resistance when the *H. capsulatum* polysaccharide immunized, whole cell immunized and nonimmunized control mice were challenged by the intravenous injection of known numbers of viable *H. capsulatum* yeast phase organism.

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Determination of Carboxypeptidase in Human Pancreatic Juice.* (24626)

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While properties and specificities of proteolytic enzymes of human pancreatic juice are well characterized, few quantitative measurements are available. Lack of data stems from 2 difficulties: obtaining samples of juice and presence of enzymes as zymogens. Human pancreatic juice is now available in adequate amount by collecting it through plastic catheters left in pancreatic duct for drainage after sphincterotomy(1). Total proteolytic activity of pancreatic juice in healthy and diseased pancreas has been determined(2). However, few data are available concerning relative amounts of trypsinogen, chymotrypsinogen, and procarboxypeptidase enzymes activated by trypsin. Procarboxypeptidase was recently isolated and studied by Neurath, *et al.*(3), although its existence as proenzyme requiring activation by trypsin had been established by Anson(4) as early as 1937. Measuremnt of carboxypeptidase in pancreatic juice is facilitated by availability of the synthetic substrate, carbobenzoxyglycyl-1-phenylalanine, first synthesized by Bergmann (5). This substrate is quite specific for carboxypeptidase and is not affected by trypsin or chymotrypsin(6). The enzyme splits off the phenylalanine moiety, liberating alpha-amino group which can be conveniently followed by ninhydrin color. The method used here is a modification of assay described by Neurath *et al.*(6). The ninhydrin reagent of Stein and Moore(7) is used for color development. The method previously described (2) for activation of total pancreatic juice proteolytic activity (utilizing calcium and minute amounts of trypsin) required 18 hours at 4°C and was somewhat variable. We devised a method utilizing commercial duodenal extract for production of enterokinase preparation which is simple to prepare, stable, and which completely activates trypsinogen of pancreatic juice in 60 to 75 minutes at room temperature. The presence of calcium ion in this extract retards autolysis of the trypsin.

Methods. These procedures were applied to measure active carboxypeptidase and total carboxypeptidase after activation of procarboxypeptidase with duodenal extract in human pancreatic juice. Pancreatic juice samples were collected from 12 patients with surgical fistulas at various intervals during their postoperative course. 1. *Reagents*—A. .1M veronal buffer (PH 7.8). B. .06M carbobenzoxyglycyl - l - phenylalanine (Mann Fine Chem.). C. 5% LiCl. D. 8% ninhydrin (Dougherty Chem. Co.) in methyl cellosolve mixed with equal volume of freshly prepared stannous chloride (16 mg dissolved in 10 ml of pH 5. citrate buffer). E. *Duodenal extract containing enterokinase*—400 mg Viodenum (whole duodenum defatted and dehydrated at low temperature, VioBin Corp., Monticello, Ill.) is suspended in 10 cc distilled water containing 200 mg $CaCl_2$ and shaken gently 18 hours at 4°C. After centrifuging, the supernatant is used as activator. This preparation is stable for at least one month if kept frozen. F. 50% N-Propanol. 2. A *standard curve* was prepared by subjecting known amounts of carboxypeptidase, varying 1.5-39 mg/per ml to procedure described below. Values were plotted against optical density. Carboxypeptidase suspension (Worthington Co.) was dissolved in 10% LiCl at 0°C. The exact amount of carboxypeptidase in this solution

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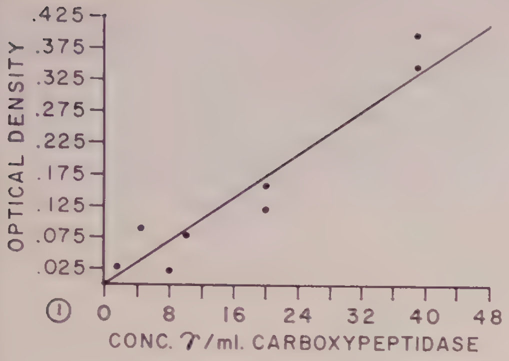

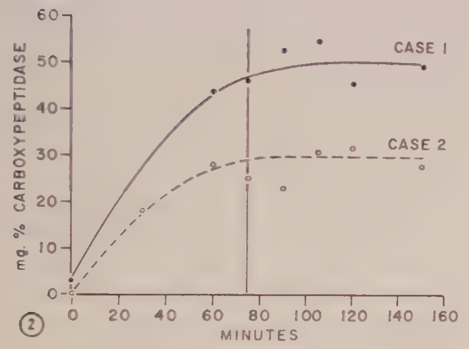

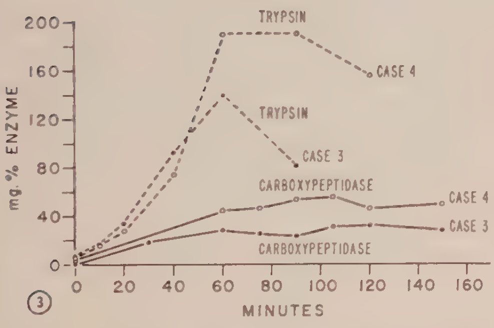

FIG. 1. Carboxypeptidase—standard curve.

FIG. 2. Activation curve 37°C. 1 ml pancreatic juice + 0.1 ml duod. extract.

FIG. 3. Comparative stability after activation.

was estimated on the basis of its nitrogen content using the value of 15.4% nitrogen cited by Neurath(3). Kinetic studies were undertaken to compare activity of standard carboxypeptidase with the highly purified preparations of Neurath *et al.*(3). A proteolytic activity coefficient, C = 13, agreed reasonably with values found by Neurath under similar conditions(8). The curve obtained was linear and first order as expected. Subsequently, all pancreatic juice values were read from the curve (Fig. 1). 3. *Procedure.* A. Activation of Pancreatic Juice. Procarboxypeptidase in pancreatic juice with 0.1 ml of duodenal enterokinase preparation at 37°C. Experiments were carried out to find optimal time of activation. Samples were taken from the activation mixture at convenient intervals and assayed for carboxypeptidase activity. The results of 2 such studies are shown in Fig. 2. B. *Incubation mixture* which contained 0.5 ml veronal buffer (pH 7.8), 0.5 ml 0.06M carbobenzoxyglycyl-l-phenylalanine, and 0.25 ml 5% lithium chloride, was brought to 37°C in water bath. At zero time 0.25 ml of enzyme solution was added. In exactly 15 minutes the reaction was stopped by rapidly pipetting *0.2 ml* of incubation mixture into a tube containing 1.8 ml water immersed in boiling water bath. After at least 5 minutes this tube was cooled and 0.2 ml aliquot pipetted into a tube graduated at 10 ml. One ml of the ninhydrin reagent was added and the tube kept in boiling water for 20 minutes. Then the tube was cooled and diluted to 10 ml with 50% n-propanol in water. This was read against a blank in a colorimeter at 550 mm. The blank was prepared by pipetting the enzyme solution into tube containing incubation mixture immersed in boiling water. After 5 minutes this was cooled and a 0.2 ml aliquot was added to 1.8 ml distilled water. 0.2 ml aliquot of this solution was treated with ninhydrin as above. 4. *Determination of carboxypeptidase in pancreatic juice.* A. Active carboxypeptidase—Since a small amount of proteolytic activity as measured by casein digestion (without activation) was present in human pancreatic juice(2), studies were undertaken to determine if active carboxypeptidase was also present. Pancreatic juice was always collected in ice bath. Freshly collected pancreatic juice was diluted 1:10 in water and assayed for carboxypeptidase A significant amount of active carboxypeptidase was found in all samples of human pancreatic juice flowing directly from pancreatic ducts. B. Total carboxypeptidase was measured by first activating pancreatic juice with duodenal

TABLE I. Active and Total Carboxypeptidase in Pancreatic Juice following Recovery from Operation.

Patient	Days post-op.	mg % carboxypeptidase Active	Total
M.L.	3	31.	57.
	10	3.4	43.
J.F.	1	113.	119.
	8	6.	
C.M.	1	14.	97.
	5	4.8	17.5
T.F.	1	38.	130.
	4	2.5	40.
E.J.	1	52.	
	3	2.5	5.
Avg, 12 patients	10+	3.9 (2.2-6.2)	52.8 (28.3–134)

extract as described above. This mixture was then diluted 1:25 and a 0.25 ml aliquot was used as the enzyme mixture.

Results. Forty determinations of active and total carboxypeptidase were made on pancreatic juice from 12 patients. Samples were collected at various intervals following operation beginning on first postoperative day. Samples collected during first 3 postoperative days showed high concentration of enzyme (Table I). In 3 instances active carboxypeptidase represented a significant part of total carboxypeptidase. In one patient a sample was analyzed on 18th postoperative day following pancreatographic study, which produces a transient inflammatory reaction(9). Active trypsin in this sample was 3.91 mg %. Normal human pancreatic juice contains no more than 0.2 mg % of active trypsin(2). Active carboxypeptidase was 119 mg % and total was 127 mg %. Evidence from pancreatographic studies and, in a few instances, direct examination at operation indicates that with proper treatment the pancreas resumes normal function 10 to 14 days after operation (9). Results of analyses of pancreatic juice collected beyond this period were considered normal, in that the pancreas had recovered from operative procedure. Analysis of 12 samples showed uniformly that there was a significant amount of active enzyme present in pancreatic juice (Table I). Average value for active carboxypeptidase was 3.9 mg %, range 2.2-6.2 mg %. Total enzyme concentration, which represents the active plus procarboxypeptidase, had average value of 52.8 mg %, range 28.3-134.0 mg %.

Discussion. Patients from whom pancreatic juice was collected were kept on naso-gastric suction for the first 3 postoperative days. In the absence of acid in the duodenum the pancreas secreted a very small amount of juice containing high concentration of enzymes. When feeding was started, the secretin produced by contact of food and acid with the duodenal mucosa, caused an increased secretion of water and bicarbonate by the pancreas, diluting the enzymes (Table I). Concentration of active carboxypeptidase was also high during this first postoperative period but diminished after a few days. Similar findings were previously noted when proteolytic activity was measured(2). High active trypsin content was correlated with presence of acute inflammation since subsidence of the acute process was accompanied by almost complete disappearance of active trypsin. High ratio of active to total carboxypeptidase found during the first few postoperative days may have been due to activation of procarboxypeptidase by active trypsin present in such cases. Thus in one patient in whose juice active carboxypeptidase was 94% of total carboxypeptidase, active trypsin was found to be markedly elevated. Values reported here for active carboxypeptidase in normal pancreatic juice were significantly higher than those reported for active trypsin which rarely rose above 0.2 mg %(2). This amount of trypsin should be adequate to activate the larger amount of carboxypeptidase found. Indications that carboxypeptidase is a rather stable proteolytic enzyme first became evident during activation studies with enterokinase. Procarboxypeptidase was almost completely activated in 60-75 minutes but little of the activity was lost during prolonged incubation at 37°C even after 3 hours. On the other hand, although trypsinogen also attained maximal activation at 75 minutes, decline thereafter was rapid due presumably to autodigestion (Fig. 3). It was also found that pancreatic juice could be kept at least 24 hours at 5°C without loss of either active or procarboxypeptidase. The stability of this enzyme and its high specificity for this type of synthetic substrate should permit its estimation in blood and urine. Accurate measurement of the blood levels of proteolytic enzymes of pancreatic origin has not been achieved. The presence of inhibitors, enzymes of similar properties and specificities, and complicated activation systems have raised doubts as to the reliability of these methods. Attempts to measure carboxypeptidase in serum and urine are in progress and preliminary studies suggest that this is possible.

Summary. 1. A method is described for determination of carboxypeptidase in human pancreatic juice. Eighteen samples of pancreatic juice gave an average value for active carboxypeptidase of 3.9 mg % with a range of 2.2-6.2 mg %. Total enzyme concentration representing active enzyme plus procarboxypeptidase gave an average value of 52.8 mg % with a range of 28.3-134.0 mg %. 2. A significant amount of active carboxypeptidase was found in fresh pancreatic juice. High values of active and total carboxypeptidase were found initially postoperatively but these declined on about the third postoperative day. 3. Carboxypeptidase was found to be more stable to autolysis than trypsin.

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Metachromatic Sulfatides in Cerebral White Matter and Kidney.* (24627)

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Of considerable biological interest is the striking metachromasia of normal myelin with toluidine blue(1). Color change of this blue dye toward red is consistent with the presence of compounds rich in negatively-charged acid groups(6). While acid mucopolysaccharides are commonly associated with metachromatic staining, it is less fully appreciated that acidic lipids may also cause intense metachromasia. These strongly charged anionic groups might be expected to bind cations. Such a process might be relevant to bioelectric potentials involved in the nerve impulse and to rapid movements of K+, Na+, Ca++, etc. across membrane systems in other body tissues.

In previous staining experiments, made *in vitro* on naturally-occurring sulfatides, it was shown that at least part of the normal lipid metachromasia of white matter may be related to sulfuric acid ester groups(2,7).

It was thus of interest to extend these staining observations to a measurement of lipid sulfuric ester groups in 2 tissues where cation transfer is biologically prominent. In the present study normal, metachromatic white matter was contrasted with normal kidney, where lipid metachromasia is negligible. Pertinent diseases which afford a range of sharply contrasting values were also included. Results bear ultimately on the intriguing function of myelin and other sulfatides in health and disease.

Materials and methods. Applicability of partitioning, separation, and identification(3, 4,8,9,11) methods used in this study were previously verified(2,7), (Witmer, F., and Austin, J. to be published). Histologically nor-

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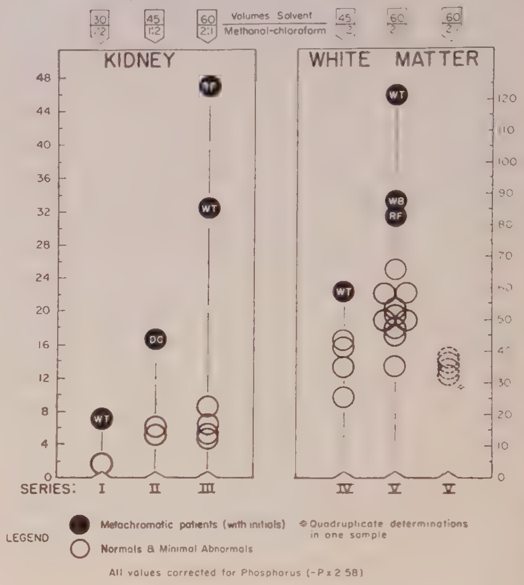
* These studies were aided by Kenny research scholarship, with the support of Nat. Inst. of Neurol. Dis. and Blindness, and Nat. Multiple Sclerosis Soc.

mal material from 7 human brains and 7 kidneys was then analyzed by identical technic with contrasting material from 6 mildly abnormal brains, 3 severely abnormal brains, and 3 severely abnormal kidneys.† Cerebral white matter was from centrum semiovale. Representative and comparable ages were used, preference given to the younger age group after myelination.‡ Normal and abnormal cases were matched as to location of white matter sample and length of fixation. Formalin fortunately maintains cerebrosides (10) and, it would appear, their sulfuric esters, or sulfatides, as well. Kidneys were capsule free. Formalin-fixed organs were homogenized, dialyzed 48 hours against water, lyophilized and dried to constant weight in a vacuum desiccator over fresh P_2O_5. Lipids were extracted from 0.5-2 g of dry powder with large volumes of chloroform-methanol mixtures. Red metachromatic lipids were traced by filter paper spot test(2) through each subsequent separation. An aqueous sodium chloride wash (730 mg %) removed water soluble materials(4). An isolation procedure was then used, reported by Lees(3) to yield a sulfatide with the chemical composition of cerebron sulfate, (referred to here as cerebroside sulfuric acid ester). Careful standardization of proportions in this procedure concentrated metachromatic lipids into one final fraction. Here they were precipitated twice with KCl in dilute methanol, and dried with N_2, P_2O_5 and vacuum. Sulfuric acid ester groups ($-OSO_3^-$) in final dry sulfatide fractions were next determined by infrared spectrophotometry at their absorption peak (8.02 μ). By double-beam infrared analysis the sulfatide isolated by this method from unfixed kidney (I, case W.T.) was spectroscopically identical both qualitatively and quantitatively with a purified reference sample of cerebroside sulfuric ester. This sample (kindly furnished by Dr. S. J. Thannhauser) was prepared by another method(5) and its spectrum is confirmatory of Lees' report(3). For purposes of convenience, lipid sulfuric acid ester (SAE) absorptions are therefore corrected by comparison with and expressed in terms of this standard cerebroside sulfuric acid ester of Thannhauser. Perkin-Elmer spectrophotometers were used with densitometer measurement, corrected for blank, of KBr discs on model 112, double beam technics on model 21, and Sartorius-Wercke microbalance weighing . Nominal corrections were made for phosphoric acid esters(5) by subtracting P x 2.58(9).

TABLE I.

CEREBROSIDE SULFURIC ESTERS

mg. per Gram (dry weight)



Results. The last 5 series of analyses contain 32 determinations. Each series is plotted separately and vertically in Table I, (I-V).

The lowest 4 values for white matter in Series IV and lowest 6 in Series V range from 25-44 mg/g and 35-51 mg/g respectively. The

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† Included in the last 2 categories are 3 brains and 3 kidneys from 4 patients with metachromatic leucoencephalopathy (ML) (#), of special interest because increased amounts of metachromatic lipids are seen histochemically in white matter and kidney tubules (2,6,7).

W. T. age 9; D.C. age 3½, (Western Reserve Hosp.); W. B. age 3½, (Mass. Gen. Hosp.); R. F. age 5, (Baylor Hosp.). (See also references 2, 6, 7.)

‡ Median age white matter from normal, mildly abnormal, and metachromatic leucoencephalopathy (M) respectively: 7, 6, 5 years; mean age 16, 15, 5 years; range 1½ - 70, 6 - 34, 3½ - 9 years. Median age kidney from normal and ML respectively: 6, 5 years; mean age 9, 6 years; range 1½ - 34, 3½ - 9 years.

only "normal" patients among these lower values are those at the extremes of age (15 months, 17 months, 70 years); the rest are mildly abnormal brains[§] (○). In series V, in the next cluster from 53-65 mg/g are healthy normals of intermediate ages 4-20. Values in ML here (●) are 127-185% those of the highest normal white matter value ($p<.05$ and $p<.005$ by extreme means test for whole series).

Normal kidneys show small amounts of SAE. Averages are 5.4-6.0 mg/g in Series II and III. These values are only 1/10 that of normal mature white matter. In strongly metachromatic kidneys from ML patients, average elevation of SAE is 3-6 times average normal (III $p<.005$). Conspicuous increases in ML cases are seen grossly by their stronger metachromasia and heavier test tube precipitation. Single-beam qualitative infrared analysis shows no essential difference between comparable normal, abnormal, ML, and reference standard sulfatides nor do double-beam high resolution technics in representative samples tested.

Discussion. The sulfatides extracted correspond histochemically, in double refraction, and in solubilities both with the diffuse lipid metachromasia of normal myelin and with excess material in brain and kidney in ML(2,6, 7).

Slightly lower than average values in normal infancy may reflect chemical immaturity of myelin with its SAE synthesis still incomplete. Reduction in old age might indicate a relative preponderance of myelin catabolism. As was found, slightly lower SAE values would also be expected in diseases with mild abnormalities or actual loss of white matter substance.

In ML, on the other hand, a paradox exists. Here, *above* normal amounts of SAE occur in brains which not only lack normal myelin, but are also lacking in "neutral fat" stages which would indicate previous myelin breakdown (7). It is likely that such a gross excess of cerebroside SAE would be a crucial departure from the normal sequences of cerebroside metabolism. In the slow but still dynamic metabolic turnover of myelin, a subtle error affecting synthesis or resynthesis may ultimately lead to inadequate myelin formation. Thus, the excess SAE found may help reconcile the apparent paradox. It is consistent with the possibility that in ML there may occur more of a gradual failure to properly build up and renew "normal myelin" in the first place, and perhaps for this reason, then, less of an apparent *de*myelination through conventional pathways.

Infrared studies contribute no evidence for the possibility that *gross* structural changes in the sulfatide unit itself might differentiate the normal and abnormal conditions studied. It will be noted that the SAE excess appears, descriptively, to constitute a "sulfatide lipidosis" which is similar to the accumulation of other naturally-occurring lipids in "lipid storage" disorders(13).

It is plausible that much higher SAE values in normal white matter, 10 times those of kidney, may reflect a relatively more important biological role for sulfatides in the nervous system.

Discussion of factors which influence phosphorus in sulfatide fractions(3) is outside the scope of this paper. Sulfatides are expressed in keeping with the existing (1955) formula for cerebroside SAE which has no P(5), but provision is made here for other formulae by recording % P found. (Range kidneys: 0.70-3.6% P in normals; 0.56-0.75% P in M.L. Range white matter: 1.27-2.38% P in normals and minimal abnormals; 0.19-0.47% P in M.L.)

Because of improvement in yields from Series I to V with technical improvements during the past 2 years, values are to be compared within each series. Extraction with greater proportions of methanol and more solvents (top Table I) gives higher yields. Duplicate determinations on duplicate samples show reasonable agreement (13%). The sparse literature on sulfatides has no strictly comparable data. If the sole normal sulfolipid value for adult mammalian white matter is corrected (x 3.3) to dry weight one figure is 39 mg/g(12). This agrees with normal SAE values obtained by more sensitive methods in the present series. It is clear that many other lipid analyses, in common with these data, are to be regarded as first-order approximations, of greater value for comparison within one controlled series than as absolute numbers. Neither this nor other current procedures neatly separate sulfatides quantitatively from nonsulfatide fractions. This factor was minimized by 1) the significant number and selected variety of simultaneously analyzed samples, 2) recording corrections made for phosphorus content, and 3) the fact that metachromatic lipids which enter the non-sulfatide fraction do so to a greater degree in ML cases where they are clearly increased in the lipid extract to begin with. Consistent biochemical, microscopic, spot test, and gross visual correlations among brains, kidneys, normals, mildly abnormals, and metachromatic cases, reproduced even with different extraction mixtures, support the coherence of the data.

[§] IV: Sudanophilic Diffuse Sclerosis, Familial Leucoencephalopathy (Minimal); V: Chronic Hydrocephalus, Familial Polioencephalopathy (2 separate cases), Multiple Sclerosis.

Summary. 1) Metachromatic sulfuric acid esters (SAE) were measured in sulfatide fractions extracted from normal and abnormal white matter and kidney. 2) Slightly lower than average SAE values for white matter were found in early childhood, in old age, and in diseases interfering with myelin metabolism. 3) The average SAE value in normal mature white matter was 58 mg/g. This was 10 times the value for normal kidney and corresponds with the much greater lipid metachromasia in white matter histologically. 4) Statistically significant elevations in sulfatide values were found in white matter and kidney in one disease (metachromatic leucoencephalopathy). 5) This accumulation may be related to an error in sulfatide metabolism, and appears, descriptively, to constitute a "sulfatide lipidosis."

It is a pleasure to thank Frank Witmer for valuable suggestions and many infrared absorbencies; Paul Amrhein for suggestions and double-beam infrared interpretations; Paul Jones for phosphorus determinations; and Drs. Lowell Lapham, E. P. Richardson, Jr., and James Hutcheson and Dept. of Pathology, for material kindly referred for study (#).

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In vivo Measurement of Radiophosphorus and Radiostrontium Absorption in Rats.* (24628)

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Hamilton(1) demonstrated *in vivo* the absorption of orally administered radioisotopes of Cl, Br, I, Na and K in humans. In the following experiments, this method has been modified to measure absorption of radiophosphorus and radiostrontium in normal adult rats. The isotopes are given by stomach tube, and the radioactivity which accumulates in the tail is measured continuously. The method avoids surgical trauma and makes it possible to measure amount and rate of absorption in individual animals under almost normal physiological conditions.

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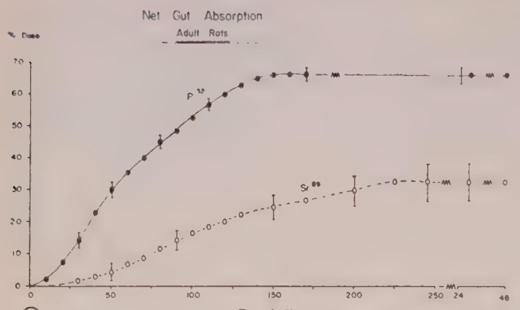
Method. 20 adult female rats of the Sprague-Dawley strain weighing 200-220 g were used. They were lightly anesthetized with urethane-barbitone, or were restrained by wrapping in a towel according to the technic described by Griffith and Farris(2). No significant difference could be attributed to use of anesthesia. The tail was inserted into a special lucite or lead holder which in turn was placed in a standard position under the counter. A thin end window Geiger-Mueller tube was used for P^{32} and Sr^{89}, and a scintillation counter for Sr^{85}. The radioactiivty in the tail was measured continuously with a counting rate meter connected to an Esterline-Angus recording milliammeter. The average diameter of the tail under the counter tube was 8.5 mm, so that a considerable part of the beta radiation from P^{32} and Sr^{89} in the superficial tissue could be detected(3), and practically all of the gamma radiation from Sr^{85}. A preliminary experiment was carried out to determine the validity of using tail radioactivity as a measure of the radioisotope in the whole body. Ten microcuries of the carrier free isotopes P^{32}, Sr^{89} and Sr^{85} were administered by stomach tube in 1 ml distilled water. The rats were killed at various time intervals up to 7 hours after a final *in vivo* measurement of the radioactivity in the tail. The intestinal tract was removed and ashed along with the feces, while the remainder of the carcass was ashed separately. Initially, the intestinal wall was washed, and radioactivity of the gut contents was determined separately, but since the radioisotope of the gut wall did not exceed 0.7% of the administered dose, it was felt that this additional step was not necessary. The quantity of radioisotope present in the carcass after removal of the intestinal tract was taken as net absorption. During *in vivo* absorption measurements tail radioactivity was measured continuously from time of administration of the isotope until the rats were killed, at the end of 3 hours in the case of P^{32}, and 6 hours in the case of Sr^{89} and Sr^{85}. They were then treated as described above, and net absorption of radioisotope was determined. From the ratio of absorbed body radioactivity (by analysis) to radioactivity actually measured *in vivo* in the tail, absorption was estimated at various times during the experimental period. Since Sr^{89} emits only beta radiation, while Sr^{85} emits essentially only gamma radiation, it was possible to make 2 distinct measurements of radiostrontium absorption in the same animal using these isotopes. The initial measurement was made with Sr^{89} as described above, and the measurement then repeated using Sr^{85} and a gamma sensitive scintillation detector with an aluminum filter of 300 mg/cm^2 to eliminate all but gamma rays. A similar procedure was used to distinguish the 2 isotopes in the ashed carcass and intestinal tract.

Results. Ratios of total radioactivity in the carcass to radioactivity measured *in vivo* in the tail have been measured. These ratios were relatively constant up to 3 hours in the case of P^{32} and 5 hours in the case of Sr^{89} and Sr^{85}, but beyond these times, the ratios become erratic. Although the relationship is empirical, it is significant that constant ratios were obtained with the beta emitting isotopes P^{32} and Sr^{89} as well as Sr^{85} which emits gamma radiation and thus suffers very little mass absorption by the tissues The ratios therefore appear to be consistent over the period of active absorption studied in these experiments. The constancy of the ratios indicate that *in vivo* determination of radioactivity in the tail gave a measure of absorption of P^{32} and Sr^{89} during the first few hours after oral administration.

Average values for absorption of P^{32} and Sr^{89} have been plotted against time in Fig. 1. Absorption of phosphate from the gut occurred very rapidly, reaching a peak within the first half hour, and very little additional absorption occurred after 2 hours. The values for 24 and 48 hours represent actual retention of the isotopes in similar groups of animals killed at these times following oral administration. In the case of radiostrontium, there was a latent period of 20-30 minutes in most animals, absorption was slower and continued for a longer time. Since the slope of the curve



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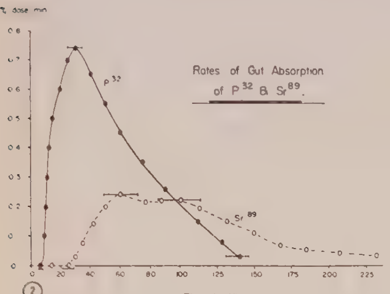

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FIG. 1. Net absorption of P^{32} and Sr^{89} in rats. Each curve is avg of 8 individual graphs. Stand. errors are shown.

FIG. 2. Rates of intestinal absorption of P^{32} and Sr^{89} derived from tangents of absorption curve. Stand. errors are indicated.

represents rate of absorption, it is possible to estimate graphically, by drawing tangents, absorption rate in individual animals at various times after administration of the isotope. These averaged values have been plotted in Fig. 2, and are expressed as per cent of the administered dose absorbed per minute.

Since some studies had required the use of anesthetic, a test was made to determine its effect. Seven anesthetized rats absorbed 32.7 ± 1.4% of oral dose, while 7 unanesthetized rats absorbed 31.4 ± 3.4% (mean ± std. error). Thus no essential difference was found. When radiostrontium absorption was measured in the same animal, first with Sr^{89} and then with Sr^{85}, some variation was observed; however in a series of 5 animals, the average absorption on the first occasion (Sr^{89}) did not differ significantly from the average for the second absorption (Sr^{85}).

Discussion. The principal advantages of this *in vivo* method are simplicity, lack of serious physiological disturbance of the animal, and more precise visualization of absorption in individual animals. The characteristic sigmoid curve obtained was similar to that observed in humans by Hamilton(1) using other ions. The difference in the rate maxima in absorption of phosphate and strontium may be due to differences in the site and gut transfer rate of these ions in different parts of the intestinal tract. Weissberger *et al.*(4) observed in the dog that P^{32} appeared in the blood within 5 minutes after oral intake, and reached a maximum at 30 minutes. This suggests active absorption high in the intestinal tract and supports the observations reported here. On the other hand, there was a delay of 20-30 minutes in most rats before any significant amounts of radiostrontium were absorbed, and the whole absorption process was slower. This delay may have practical significance in the case of accidental ingestion of radiostrontium, since it indicates a period during which prompt treatment might be effective. However, most of the absorption of radiostrontium which did take place occurred within the first 5-6 hours, as was observed by Jones and Coid(5). The use of this short period allowed more direct measurement of absorption by reducing endogenous Sr excretion to 2.1% of oral dose.

The double tracer technic, using Sr^{89} and Sr^{85}, provides a means of comparing strontium absorption in the same animal under normal and then under experimental conditions. It should prove useful for studying treatments designed to reduce radiostrontium absorption, or for investigating factors which may modify it.

Summary. 1. An *in vivo* method has been described for studying radiophosphorus and radiostrontium absorption from the intestinal tract in rats. The method is simple, avoids surgical trauma and permits visualization of the absorption process in individual animals. Rate of absorption at various times may also be estimated. This is not possible with the standard balance studies. Anesthesia is not necessary, though no difference of Sr^{89} absorption was found when anesthesia was used. 2. Absorption of radiophosphorus was very rapid, reaching a peak at 30 minutes. In most animals, there was a delay of 20-30 minutes be-

fore significant amounts of radiostrontium were absorbed, and the whole process was slower. 3. It was possible to measure radiostrontium absorption on two different occasions in the same animal, first with Sr^{89} and then with Sr^{85}. This should prove of value in studying factors affecting strontium absorption.

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Evaluation of Laboratory Diagnostic Procedures with A/Asian Influenza.
(24629)

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The recent influenza epidemic stimulated many virus diagnostic laboratories to determine whether recommended laboratory procedures were adequate and to evaluate alternative diagnostic procedures(1). Throat washings and serum specimens we received were processed by standard procedures, and results analyzed to investigate the following: 1) comparison of complement fixation and hemagglutination-inhibition tests for routine diagnosis, 2) relationship between time of appearance of antibodies titrated by above methods, 3) correlation between increased antibody titers and virus isolation, and 4) comparison of several isolation methods.

Materials and methods. The majority of acute and convalescent serums were obtained from individuals 10 to 65 years of age. Samples were submitted from many areas of the United States. Selection of patients and collection of throat washings were made by numerous physicians and health officers with varying degrees of experience. Due to this fact, specimens were often subjected to suboptimal conditions for storage and transportation. Infected allantoic fluids served as specific antigens in both serologic tests. These were produced by injection of approximately 10,000 EID_{50} by the allantoic route in 11-day embryonate eggs. Following 48 hours incubation at 37°C, the eggs were chilled several hours, fluids harvested, clarified by low speed centrifugation and stored at 4°C. Virus strains used were A/Asian/Japan/305/57, A/Denver/1/57, and B/GL/1739/54. *Serologic procedures.* Complement fixation (CF) test employed was the standard technic in the Virus and Rickettsia Section. With this method, 0.1 ml of serum and antigen dilutions are mixed with 0.2 ml of complement and later sensitized sheep erythrocytes to give a final total volume of 0.6 ml. Optimal dilutions of antigen as determined in box-type titrations were used. Complement titers were measured in presence of each antigen, and dilutions contained 2 optimal units. The test was incubated overnight at 4°C followed by 20 minutes at room temperature, at which time sensitized cells were added. The test was read after incubation at 37°C for one-half hour. The tube containing greatest dilution of serum in which less than 50% hemolysis (estimated) occurred was considered as the positive end point. Hemagglutination-inhibition (HI) tests were performed as previously described

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(2,3) with the modification that serums were heated at 56°C for 30 minutes and then treated with M/90 solution of potassium periodate to destroy nonspecific inhibitors. One volume was mixed with 2 volumes of periodate and held overnight at 4°C. Two volumes of 1% glycerol saline were then added to stop the reaction of the periodate(4). *Isolation procedures.* Penicillin and streptomycin, approximately 200 units and 100 mg/ml respectively, were added to aliquots of each throat washing before inoculation. All throat washings were inoculated into the amniotic cavity of 11-day-old embryonate hens' eggs. A number of suspected throat washings were inoculated into 3 tissue culture tubes of monkey kidney cells and a few were tested in HeLa and human amnion cells. Each test system received 0.1 ml inoculum. At least 2 transfers were completed before a specimen was considered negative.

Results. To compare efficacy of 2 recommended serologic methods, results were analyzed from 422 cases in which diagnosis could be made on the basis of a 4-fold or greater increase in antibody measured by one or both test methods. Analysis of positive results is presented in Table I. Only a little over half of the cases were positive by both tests (56%). While an additional 33% were positive by the CF test alone, only an additional 11% were positive by HI test alone. The question arose as to whether these differences might relate to the convalescent period when serum was collected. The data were examined with this in view, and the results listed in Table I.

TABLE I. Relationship between Time of Collection of Serum and Serologic Results.

Days after onset*	Total positive cases†	CF & HI No.	CF & HI %	CF only No.	CF only %	HI only No.	HI only %
7- 9	23	7	30	12	52	4	17
10-12	57	22	39	34	60	1	2
13-15	165	89	54	58	35	18	11
16-18	63	38	60	16	25	9	15
19+	114	79	69	19	17	16	14
Total	422	235		139		48	
	100%	56%		33%		11%	

* Acute phase specimens were drawn during first week of illness. Days after onset indicate interval before collection of convalescent serum.

† In all cases 4-fold or greater increase in antibody titers were demonstrated by one or both methods.

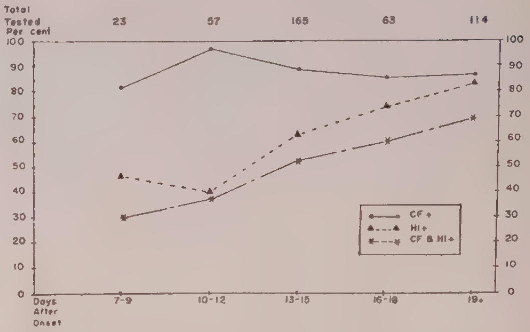

FIG. 1. Influence of interval between onset and convalescent serum specimens on rates of diagnosis by CF and HI methods in 422 serologically proven cases of influenza infections.

When specimens were collected 10 to 12 days after onset of illness, approximately 60% of the cases diagnosed by CF were missed by the HI test. In contrast, the marked disparity between results with the 2 serologic procedures was not evident when convalescent serums were collected 19 days or longer after onset, at which time approximately 70% were diagnosed by both methods. It is evident, however, even after the longer interval, that some cases were detected by one method and not the other. It is desired to point out that the total cases diagnosed by the CF test at a particular time interval varied between 82 and 99%, whereas total cases diagnosed by the HI test varied between 41 and 83%. Those cases where diagnosis was made by HI test and not by CF test may have been because the acute phase serum was obtained when CF antibody level was elevated and did not rise further. Relative differences in sensitivity between CF and HI procedures are contrasted in Fig. 1. With an increasing number of days after onset, results obtained by both tests become nearly parallel. The obvious conclusion is that the CF test would be preferred when convalescent serum was collected early after onset, whereas if the HI test alone is to be employed the convalescent serum specimen should be collected 3 weeks after onset. It must be concluded that the greatest efficiency of diagnosis results when both methods are employed. From a practical standpoint the CF test would be used for routine work and when CF test results are negative, use of the HI test is indicated.

Isolation attempts. A total of 530 throat washings and specimens of lung from autopsies were tested and from these 102 isolates (19.2%) were obtained by usual chick embryo technics. In one limited study of 43 successful isolations, 19 were positive and typed from the first egg inoculation, whereas 24 required a transfer in additional eggs. In 29 positive cases, it was found that in only 2 cases were allantoic fluids harvested with hemagglutinin titers suitable for typing when 0.1 ml of throat washing was inoculated into both amniotic and allantoic cavities. Negative allantoic fluid required one or 2 additional transfers before all could be typed.

With 102 specimens, a comparison was made of relative sensitivities between standard chick embryo amniotic isolation technics and hemadsorption procedure developed by Vogel and Shelokov(5). These specimens were tested simultaneously, and 35 were positive by one or the other procedure. Only 3 specimens yielded virus by monkey kidney tissue culture hemadsorption method, so that this method was definitely less sensitive in our laboratories. On the other hand, 2 of the 3 positive by tissue culture method were missed by egg inoculation procedure.

In addition to inoculation of above described tissues, other cell lines (HeLa and human amnion) and mice were also inoculated. No isolations were successful in any of these systems. Several blind passages were made in each case.

Attempts to correlate virus isolations with positive serologic diagnosis were seriously hampered by the fact that both kinds of specimens were not usually obtained from the patient. In only 67 cases were throat washings submitted with acute and convalescent serums. There were 42 cases diagnosed by either method and 16 cases demonstrated the presence of virus in their throat washings. From 16 cases where influenza virus was isolated, 15 were positive by the complement fixation test, while 8 were positive by the HI test. In 26 cases from which no virus was isolated but positive serologic diagnosis was made, rates of diagnosis by CF or HI tests were similar to those found with positive virus isolations. These results indicate greater difficulty in isolation of virus from specimens than might be expected if patients were carefully selected, and if throat washings were properly collected and transported. Under these circumstances, however, positive serologic support of a diagnosis is much more reliable than virus isolation attempts.

Discussion. As part of an extended evaluation of laboratory diagnostic procedures currently in use for recognition of influenza, the following conclusions seem warranted. The CF test appears preferable to the HI method for routine diagnosis because antibodies measured by CF test often appeared in elevated concentrations approximately 10 days before HI antibody levels were increased. Furthermore, the difficulties encountered with inhibitor sensitive strains of influenza rule out the HI test for many inexperienced laboratories. In addition, a strain specific CF test(6) may yield information similar to that obtained by the HI test without the disadvantage of having to utilize serum treatment procedures for removal of nonspecific inhibitors. The more rapid rise in CF antibody titer apparently results from an anamnestic response to antigens which are not concerned in the virus neutralizing or hemagglutinin inhibiting reaction. The slower response as measured by the HI test must be that resulting from the first experience with a new set of antigens. If this is correct, then a more parallel response could be expected with CF and HI tests in epidemics which occur when closely related viruses had been prevalent for several years. It is possible, therefore, that the increased advantage of the CF test would be evidenced only during epidemics such as the one just experienced. In spite of the need for rapid diagnosis, our data made it clear that the embryonate egg remained the most sensitive host for attempts at isolation of influenza viruses. More recent modifications reported by Shelokov *et al.*(7), may result in increased efficiency of the new method.

Summary. For routine serological diagnosis of influenza, the CF test is preferable to HI test. A/Asian influenza virus was more

readily isolated in the embryonate egg than in monkey kidney cell tissue culture.

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Factors Related to Psittacosis Virus (Strain 6BC) Growth. V. Folic Acid-like Factor in Infected Cells.[*] (24630)

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Inhibition of growth of the 6BC strain of psittacosis virus by sulfonamides and reversion of this action of sulfonamides in a competitive manner by p-aminobenzoic acid and pteroic acid and in noncompetitive manner by folic acid or citrovorum factor led Morgan to suggest(1) that the cell infected with psittacosis virus might be capable of synthesis of folic acid from precursors. If this is true, one would expect that folic acid content of viral-infected cells would be higher than that of noninfected cells, and studies were initiated to determine if it was possible to detect such net increase in folic or folic acid-like factors in viral-infected cells. L cells were chosen since these cells require folic acid(2) and they support growth of psittacosis virus when cultivated in a semi-synthetic medium(3). These experiments indicate that L cells infected with psittacosis virus contain increased amounts of folic acid or a folic acid-like factor.

Materials and methods. The mouse fibroblast cell line, strain L, obtained from Dr. Wilton R. Earle, Nat. Cancer Inst., Bethesda, was maintained in T flasks[‡] in growth medium consisting of Earle's balanced salt solution; 0.06% yeast extract[§]; 0.18% lactalbumin hydrolysate[||]; and 10% horse serum. Semi-synthetic medium (SSM) was a modification of medium described by Bader and Morgan(3) in which folic acid was omitted, concentration of p-aminobenzoic acid (PABA) was doubled, and 1% dialyzed horse serum was added. The egg-adapted strain of psittacosis virus (6BC) was used, as described previously(1,4). To minimize folic acid content of virus inocula, virus stock was prepared from virus grown in L cells which were maintained in SSM for 8 days before infection. After infection, daily microscopic observations of cells was carried out until a definite cytopathic effect of the virus was noted; then the flasks were frozen and thawed slowly to break up the cells and the entire culture harvested and stored in a dry-ice chest for use as stock virus which was diluted in SSM as needed. Sulfadiazine (SDZ) was used as a sterile solution of sodium salt. P-aminobenzoic acid (PABA) was dissolved in distilled water as the sodium salt and sterilized by filtration. Folic acid determinations were carried out according to the method adopted by Assn. of Official Agricultural Chemists(5), using *Streptococcus faecalis* as test organism (ATCC #8043). Chicken pancreas extract was used as source of glutamic acid conjugase. Bacterial growth was measured turbidimetrically with Bausch

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[†] Present address: Nat. Inst. Dent. Res., Bethesda, Md.

[‡] Obtained from Kontes Glass Co., Vineland, N. J.

[§] Obtained from Difco Labs., Detroit, Mich.

[||] Obtained from Nutritional Biochemicals Corp., Cleveland, O.

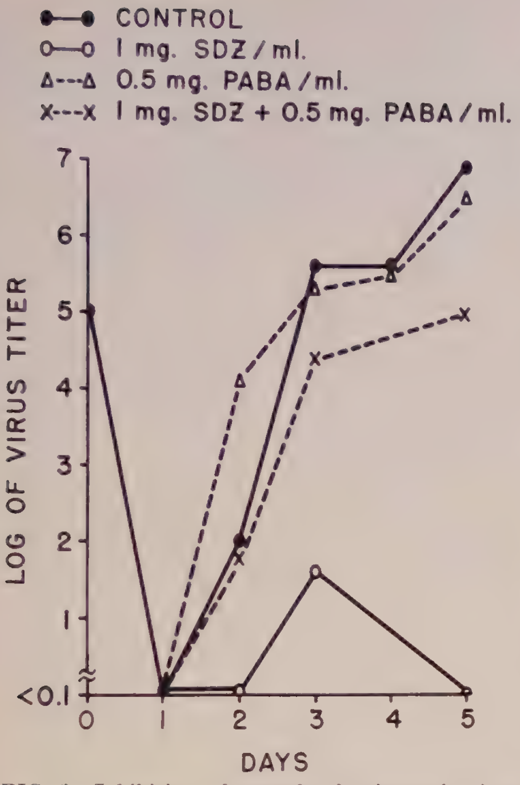

FIG. 1. Inhibition of growth of psittacosis virus in L cells by SDZ and its reversal by PABA.

and Lomb spectrophotometer at 5,000 Angstrom units.

Results. Inhibition of viral growth in L cells by SDZ and its reversion with PABA. Because of reported variability(6,7) in sulfonamide sensitivity *in vivo* of members of lymphogranuloma-psittacosis group of viruses, depending upon host used, it was of interest to determine if the 6BC strain used was sensitive to SDZ in L cells and if inhibition could be reversed by PABA.

L cells, cultivated in T-15 flasks in growth medium and infected with the virus, were divided into 4 groups and media containing compounds noted in Fig. 1 were then used in each flask. The fluids were removed daily for 5 days and titrated for virus content, and media containing respective drugs in the described concentrations were added to each flask. PABA (0.005 mg) was injected into each egg with the virus inoculum to counteract possible growth inhibition by SDZ carried over from tissue cultures(1,4). Results are shown in Fig. 1. Each curve represents the geometric mean of titers of 3 identical experiments. SDZ inhibits virus growth in L cells and PABA has the effect of reversing this inhibition. PABA alone had no significant effect on growth of virus.

TABLE I. Folic Acid Content of Infected and Noninfected L Cells.

		Virus titer of inoculum, LD_{50}/ml	Cytopathic effect, days	Folic acid (mμg)		
Exp.	Flask			Total	Inoculum	Net
I	A	3.9	9	426.4	.0	426.4
	B	0	0	202.8	2	200.8
II	A	4.0	9	431.6	.0	431.6
	B	0	0	213.2	5	208.2

Folic acid content of L cells infected with virus. When a uniform monolayer of L cells had covered the bottom of T-60 flasks, each flask was changed to the SSM, using 10 ml/flask. Cells were then maintained in this medium with complete renewal of fluids daily for 14 days to deplete them of folic acid. Two flasks were chosen and flask A was inoculated with living virus, while flask B received a similar quantity of heated virus. Folic acid assays were made of media containing the virus inoculum and only barely detectible quantities of folic acid were found in the heated virus. On the following day, the fluids were removed, stored at –40°C, and replaced with SSM. During remainder of experiment, depending on pH of cultures, the media on both flasks were removed, stored, and replaced with SSM. On the day the infected flask began to exhibit cell degeneration due to cytopathogenic effect of the virus, both flasks were frozen and thawed, and the entire contents of each flask were harvested and pooled with the respective fluids taken off on previous days, concentrated by lyophilization, and assayed for folic acid with results shown in Table I.

These data demonstrate that materials from the cultures infected with psittacosis virus contain twice as much folic acid as the uninfected cell cultures.

Discussion. Susceptibility of viruses in the lymphogranuloma venereum-psittacosis group to sulfonamides seems to indicate that these agents either possess enzyme systems of their own or are capable of inducing formation of such new systems essential for viral replication in host cells following infection. Folic acid is essential for replication of psittacosis virus, as indicated by viral growth inhibition by sulfonamides(1) or folic acid analogues(8) in chick embryo tissue *in vivo* or *in vitro* in amounts not toxic for host or its isolated cells. It appears that quantities of folic acid available in the cells are not adequate for growth of psittacosis virus and that synthesis of this essential factor takes place in the infected cell. The fact that cells depleted of folic acid for 14 days still support viral growth, further emphasizes this probable folic acid synthesis.

Increase in folic acid in L cells infected with psittacosis virus lends further support to the suggestion that such infected cells are capable of folic acid synthesis(1). Since folic acid has been shown to be a vitamin requirement for L cells(2), it is unlikely that these cells possess such synthetic capacity, but rather that this property is due to an enzyme or enzyme system acquired as a result of virus infection. Whether this synthetic activity is part of the intact virus particle itself or can be demonstrated only in the infected host cell remains to be determined.

Recent evidence indicates that folic acid or allied compounds are specific constituents of the psittacosis group(9), and the analyses on virus inocula showed some folic acid in the heated preparations. Furthermore, direct evidence has been presented that viruses in this group do possess enzymatic activity with the demonstration by Allen and Bovarnick(10) of reduced diphosphopyridine nucleotide cytochrome c reductase activity in purified preparations of meningopneumonitis virus, though other investigators(11,12) have failed to demonstrate enzymatic activity for viruses of this group. However, as Cohen(13) has pointed out, random testing for enzymes in viruses has little chance of success unless experiments are designed to test for those metabolic systems which may be related to the particular pattern and site of development of a given virus. Sulfonamide sensitivity of psittacosis virus (6BC) presents a guide to such a system, *i.e.*, folic acid synthesis, though the enzymatic mechanism remains unknown.

Summary. Growth of psittacosis virus (6BC) in L cells *in vitro* was inhibited by sulfadiazine and this inhibition was reversed by addtion of PABA. L cell tissue cultures infected with the virus contained 2 or more times the amount of folic acid in noninfected cultures. The possibility of introduction into the cell or induction in the cell by the virus of enzyme or enzyme system, making the infected cell capable of folic acid synthesis, is discussed.

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Relationship of Vit. A and Carotene to Bovine Serum Proteins.*† (24631)

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The relationship of circulating blood carotenoids to serum proteins was recognized in 1914 by Palmer and Ekles(1). More recently, specificity of the affinity of certain serum and lipoproteins for both Vit. A and carotene have been investigated. In human serum, Crook and El Marsafy(2) reported that carotene was primarily associated with beta globulin and albumin fractions when serum proteins were separated electrophoretically, while Krinsky, *et al.*(4) using different technics found that 90% of circulating carotenoids were bound to alpha and beta lipoproteins. However, these lipoproteins bound only a small fraction of circulating Vit. A. Using ammonium sulfate fractionations of human serum proteins, Dzialoszynski, *et al.*(3) concluded that Vit. A and carotene were associated with albumin. A hypothesis has been advanced that binding capacity of serum proteins aids in thwarting electrolytic destruction of Vit. A and carotene. The quality of dietary protein for rats has been shown to alter utilization of dietary carotene(5). This phenomenon may have been mediated through alteration of type and quantity of protein in serum. This investigation was initiated to elucidate association of carotene and Vit. A to specific serum proteins in the bovine animal. Further, alteration of serum proteins by Vit. A deficiency was studied.

Method. In vitro. Proteins from 30 ml of serum, from a Guernsey cow, were separated in Spinco continuous-flow electrophoresis apparatus into 16 fractions at room temperature (75°F) at the rate of 1 ml/hour with sodium veronal buffer (pH = 8.6; u = .02) (90 ma; 1000 volts). Electrophoretic mobility of lyophilized protein fractions on paper strips was compared to original serum sample. Vit. A and carotene were extracted in light petroleum ether from protein fractions previously treated with ethanol. Because of apparent loss of Vit. A, a second electrophoretic separation of serum proteins, from the same cow, was performed in a dark refrigerated room (36°F). The procedure differed only in amount of current applied (60 ma; 650 volts). Protein fractions were saponified in alcoholic KOH prior to extraction of Vit. A and carotene in light petroleum. Identification and relative concentration of carotene in the protein fractions were performed by spectrophotometric analyses while Vit. A was determined by Carr Price reaction.

In vivo. Two identical twin Holstein heifers, approximately 20 months old, were fed carotene-free ration of straw, grain, and cottonseed meal until Sept. - (150 days). One million I.U. of Vit. A were administered orally *via* capsule to animal "B". At this time electrophoretic patterns of serum proteins in both heifers were similar. On Sept. 23, animal "A" exhibited acute Vit. A deficiency syndromes manifested by blindness in one eye and anasarca in both fore and hind legs. Blood was withdrawn from both heifers for electrophoretic separations of plasma and serum proteins 3 days following onset of deficiency syndromes. An aqueous emulsion of beta carotene was prepared with Tween 80 (3 mg/ml) and intravenously administered to both heifers in accordance with body weight (10 mg/100 lb). Blood was withdrawn prior to and at 5 minutes, 2, 4, 6, 8, 12, 24, 48, 72, 96, 120, and 144 hours following carotene administration for plasma carotene and Vit. A analyses(6). Five days following carotene injection, serum and plasma from both heifers were subjected to electrophoretic separations of proteins. An aqueous emulsion of Tween 80 and Vit. A acetate was intravenously injected into both heifers(10 mg/100 lb body weight). Blood samples were withdrawn at 4 and 10 days following, and plasma and serum proteins were separated electrophoretically. Electrophoretic separations of plasma and serum proteins of the heifers were per-

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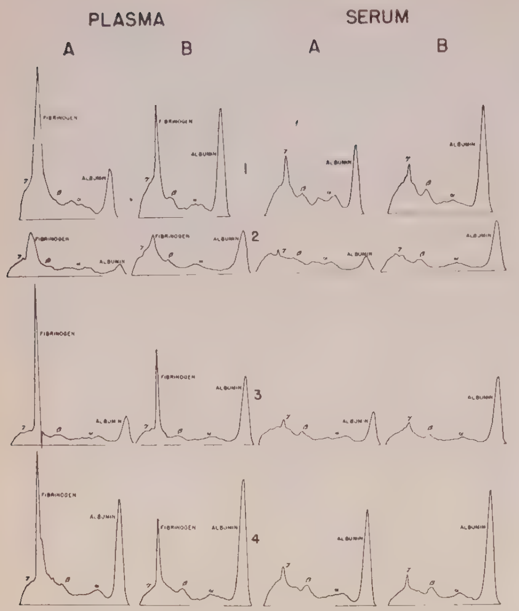

FIG. 1. Alteration of plasma and serum protein fractions as influenced by vit. A deficiency. A = deficient; B = normal. No. 1 and 2 = progressive vit. A deficiency; No. 3 and 4 = recovery following vit. A administration.

formed on paper strips (0.006 ml) in Durrum type cell with sodium veronal buffer (pH = 8.6; u = .075) for 14 to 16 hours.

Results. In vitro. Initially, bovine serum proteins were electrophoretically fractionated at room temperature. Carotene was associated with bovine serum proteins in the following percentages: albumin, 75.1; alpha globulin, 6.8; beta globulin, 8.9; and gamma globulin, 9.2. Vit. A could be detected only on albumin. Much of Vit. A and carotene was degradated during fractionation. Therefore, the second elctrophoretic separation of serum was performed in a dark refrigerated room. The low temperature necessitated use of less current (60 ma). This fractionation resulted in undefined separation of albumin and alpha globulin. All protein fractions were saponified and Vit. A and carotene were extracted in light petroleum. Only 2% of total carotene was found in fractions other than albumin and alpha globulin. While no Vit. A was associated with gamma globulin, beta globulin possessed 15% and albumin-alpha globulin fraction contained 85% of total amount of serum vitamin. These results support the evidence of others such as Ganguly, *et al.*(7), that Vit. A and carotene are bound to specific proteins in serum. *In vivo.* Growth patterns, hair swirls, color markings and blood typing were used to establish that heifers were identical twins. Further, electrophoretic separations of serum protein of both animals on Sept. 1 were similar. On Sept. 26, three days following onset of acute Vit. A deficiency syndromes in animal "A" (blindness and anasarca) serum albumin in the deficient heifer was lowered (Fig. 1, No. 1). Since our previous *in vitro* investigation revealed that albumin bound the majority of circulating carotene in sera, similar amounts of carotene (based on body weight) were intravenously administered to both deficient "A" and normal "B" heifers. Disappearance rates of plasma carotene revealed that the deficient animal maintained approximately half the plasma carotene level of the normal heifer(Fig. 2). Probably this phenomenon was related to low serum albumin level. Ganguly, *et al.*(7) hypothesized that specific serum proteins may be responsible for absorption of Vit. A and carotene. This thesis would lend support to recent study by Diven and Erwin(8) which indicated that Vit. A-deficient sheep lost in part the ability to utilize dietary carotene. Plasma Vit. A analyses substantiated the findings of Church, *et al.*(9), that the mature bovine was unable to convert metabolic carotene to Vit. A. On Oct. 2, electrophoretic separation of plasma and serum protein (Fig. 1, No. 2) showed that albumin content was further depressed. Thus, metabolic carotene did not alleviate ab-

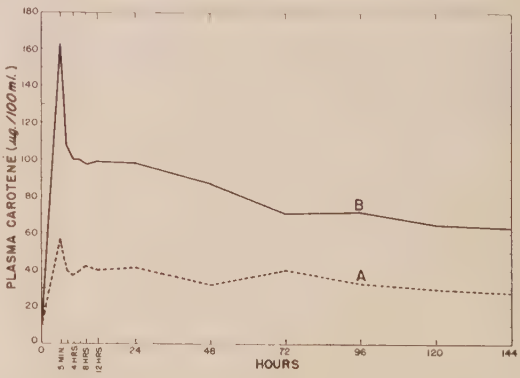

FIG. 2. Effect of vit. A deficiency on carotene capacity of bovine plasma. A = deficient heifer; B = normal heifer.

normal serum protein patterns. Four days following intravenous Vit. A acetate administration, Vit. A deficiency syndromes in heifer "A" disappeared and albumin content of sera was elevated (Fig. 1, No. 3). Electrophoretic separations of sera 6 days later (Fig. 1, No. 4) showed elevation in serum and plasma albumin that approximated normality. These results further exemplify that metabolic carotene in the bovine possesses little value in alleviating syndromes predisposed by Vit. A deficiency.

Summary. Bovine serum protein was fractionated with a continuous flow electrophoresis apparatus. Vit. A and carotene were principally associated with albumin. When one co-twin of identical twin heifers was subjected to Vit. A deficiency, serum albumin was progressively reduced. Following an intravenous injection of carotene, the deficient heifer low in serum albumin retained approximately one-half the plasma-carotene concentration as her normal co-twin. Serum albumin returned to normal in the deficient co-twin within 10 days following intravenous Vit. A administration.

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Effect of Corticotrophin on Guinea Pig Plasma Corticosteroids.* (24632)

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In view of the similarity between the major blood adrenocortical steroids of man and guinea pig we became interested in using the guinea pig for preclinical assessment of corticotrophins and repository media. Liddle, *et al.*(1) have used the 24-hour urinary steroid output of this species as a method for evaluating effectiveness of corticotrophin. We decided to explore the plasma steroid concentration as possibly offering a more rigorous and rapid evaluation procedure.

Material and methods. Male guinea pigs weighing 350-400 g were used. The animals were sacrificed under light ether anesthesia and exsanguinated from the jugular vein with heparin as the anti-coagulant. Plasma corticoids were determined by the method of Porter and Silber(2). Highly purified corticotrophin (100 USP units/mg) was dissolved in the various menstrua tested *viz.*, 15% gelatin; 4% phosphorylated hesperidin plus 15% gelatin; saline. All injections were given subcutaneously.

Results. Fig. 1 presents distribution of plasma corticoid values of a group of 50 untreated animals. Average value is 43.4 μg/100 ml plasma. These values were further analyzed to determine whether a seasonal variation in plasma steroid concentration existed, but no significant difference was found in values of animals sacrificed in January, June, or September (Fig. 1). The base values are in agreement with those of Done, *et al.* (3) and Boulouard(4) who used a procedure similar to ours for sacrificing their animals. Sobel, *et al.*(5) have also found that the stress produced by ether anesthesia and surgery, such as exposure of the carotid artery

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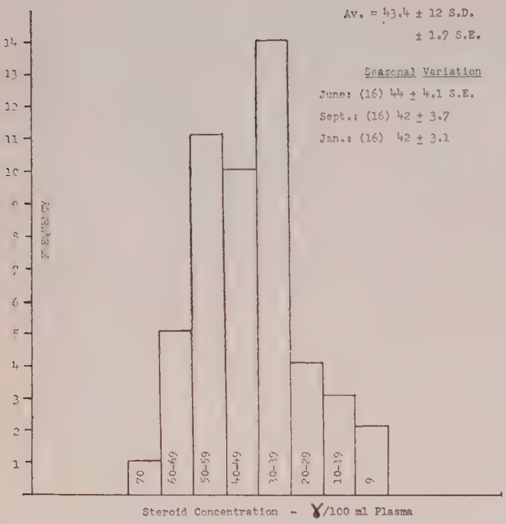

FIG. 1. Distribution of plasma 17-OHCS values of untreated male guinea pigs. 350-400 g body wt.

was insufficient to induce increased release of ACTH, as measured by urinary corticoid output. However, Eik-Nes(6) reported the average plasma steroid value of guinea pigs to be 24 μg/100 ml when a special neck clamp was used for sacrifice whereas animals anesthetized before bleeding had a higher concentration of plasma corticoids.

The marked diurnal variation in plasma 17-OHCS content of humans prompted us to examine this phenomenon in the guinea pig. Our data indicate that at least within the 8-hour period (8 a.m.-5 p.m.) no variation in blood steroid content is evident, in contrast to Migeon's(7) data which demonstrates that in the human a marked decrease in plasma corticoids occurs between these times. Our observations agree with the findings of Schweitzer (personal communication) who noted that the eosinophil count of the guinea pig does not vary during this time period, and also with those of Lazo-Wasem and Hier(8) who collected 8 hr. guinea pig urine samples from 8 a.m.-12 M and could detect no difference in steroid output during this time.

Since the procedure for determining corticoids in plasma measures only unconjugated steroids, representative plasma samples of different steroid content were examined to determine whether any glucuronide conjugated steroid was present. We noted that after enzymatic hydrolysis with β-glucuronidase a small but consistent amount of glucuronide conjugated Porter-Silber chromogen is present in most samples examined. This is in marked contrast to the large amount of conjugated steroid found in human plasma. The significance of this data is admittedly questionable and does not preclude the presence of conjugates other than glucuronides. Péron and Dorfman(9) report that all urinary corticoids of guinea pigs are present in the free form, but Wyngaarden(10) has noted that C^{14}-hydrocortisone administered to guinea pigs results in excretion of a small percentage of urinary metabolites as glucuronides. This has also been noted by Silber & Porter(11).

The possibility of establishing a quantitative relationship between plasma corticoid content and dose of administered corticotrophin was next considered. The data presented in Table I show that a maximum increase in plasma corticoid occurs 3 hours after subcutaneous injection of corticotrophin dissolved in gelatin, with a return to normal values between 8 and 12 hours later. This phenomenon is the same regardless of dose administered, although a difference in rate of decay is evident.

With effective delaying agents it is possible to alter this pattern so that 12 hr after administration of the hormone, increased amounts of plasma corticoids still persist. Table II demonstrates that addition of phosphorylated hesperidin to corticotrophin in gelatin, results in maintenance of an increased concentration of plasma corticoids 12 hr after administration of the hormone, whereas after a similar dose of corticotrophin dissolved in gelatin alone, the steroid values have returned to normal. This is attributed to ability of phosphorylated hesperidin to effect both a

TABLE I. Plasma Levels of 17-OHCS following Corticotrophin Injection.

Dose, USP units	Hr after ACTH inj.* 0	3	8	12
1.5	49 ± 2 (5)	144 ± 14 (5)	61 ± 4 (5)	41 ± .9 (5)
7.0	49 ± 2 (5)	183 ± 10 (6)	131 ± 16 (8)	35 ± 3 (8)

( ) No. of animals.
* ACTH dissolved in 15% gelatin.

TABLE II. Effect of Retarding Agents on Steroidogenic Effect of ACTH.

	Plasma 17-OHCS, μg/100 ml	
Hr after inj.	15% gelatin	15% gelatin + 4% P.H.*
0	43 ± 2	43 ± 2
3	184 ± 12	200 ± 14
8	132 ± 8	198 ± 15
12	35 ± 2	124 ± 9
24	41 ± 3	46 ± 5

Dose of corticotrophin = 10 USP units.
* Phosphorylated hesperidin.

slow release of corticotrophin into the blood stream and protection against inactivation of the hormone at site of injection. These data are in accord with those reported for ACTH in phosphorylated hesperidin plus gelatin in the rat, using adrenal ascorbic acid depletion as a measure of effectiveness(12).

Table III presents data comparing the effect of corticotrophin dissolved in gelatin, and in saline on plasma steroid concentration of the guinea pig. It is apparent that the effect of ACTH in gelatin is superior to that of the hormone dissolved in saline. This is in accord with clinical results in the human.

Finally, quantitation of response was examined. When the plasma steroid concentration obtained 3 hours after injection of ACTH in 15% gelatin is plotted against the log of the dose of corticotrophin administered, a straight line is obtained indicating the suitability of this parameter for assay purposes (Table IV). The statistics for the dose response data indicate excellent precision and sensitivity.

TABLE III. Guinea Pig Plasma Steroid Values after Administration of Corticotrophin* in Saline Solution and in 15% Gelatin Solution.

	μg/100 ml plasma	
	15% gelatin	Saline
Exp. 1	189	78
	156	107
	173	94
	153	97
	162	103
Avg	166 ± 6.6	96 ± 5.9
Exp. 2	160	133
	147	122
	170	102
	159	98
	133	110
Avg	154 ± 6.3	113 ± 6.4

* 1.5 USP units ACTH administered subcut. Blood samples obtained 3 hr after inj.

Conclusion. The plasma corticosteroid content of the guinea pig offers a suitable means for evaluating the potency of adrenocorticotrophic hormone, and also the menstrua which may be useful in extending the physiological or pharmacological effect of this hormone.

Summary. The response of the guinea pig to exogenous adrenocorticotrophin was determined using plasma steroid concentration as criteria of response. Average steroid concentration for 50 untreated male guinea pigs was 43 ± 1.7. A graded response was obtained after corticotrophin administration which when plotted yielded a straight line with a slope adequate to determine differences between a 1½ to 3-fold increment in dose. Comparison of corticotrophin administered subcutaneously in a gelatin menstruum with that administered in water showed a 2-3 time greater activity when gelatin is employed as a menstruum than when the hormone is dissolved in water. This is in accord with findings in the rat using the adrenal ascorbic acid depletion as a measure of activity, and clinically in the human. Small amounts of glucuronide conjugated corticoids were also found in the plasma.

TABLE IV. Relationship between Dose of Corticotrophin* and Response Elicited.

No. pigs	Dose, USP units	Response, γ 17-OHCS/100 ml plasma
14	.50	82 ± 6.7
14	.75	114 ± 4.3
14	1.50	145 ± 5.9

b = 193 ± 19.4
s = 21.09
λ = .109

* Corticotrophin dissolved in 15% gelatin and administered subcut. Animals sacrificed 3 hr after inj.

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Composition of Renal Lymph and Its Significance.* (24633)

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Various concepts of the function of renal lymph have been evolved, based largely on indirect evidence(1). The present report deals chiefly with electrolyte concentrations in renal lymph, preliminary data from a study designed to obtain more direct and definitive evidence of its function.

Procedure. Healthy mongrel dogs of both sexes were anesthetized with sodium pentobarbital (25 mg/kg). A polyethylene catheter was inserted into jugular vein *via* 15 gauge needle and utilized for plasma sampling and administration of fluids and drugs. The thoracic duct was cannulated and thoracic duct lymph collected in most experiments. Both ureters were catheterized at their junction with the bladder and urine collected separately from each kidney. Femoral arterial pressure was recorded with a mercury manometer. A left flank incision was employed to expose the kidney. The fat at both poles was usually ligated to engorge the capsular lymphatics and permit their cannulation with #50 polyethylene tubing. Renal lymph was collected in small glass tubes capped with one-hole stoppers to prevent evaporation and volumes estimated to the nearest .05 ml. During the prolonged period of exposure the kidney was covered with saran wrap to prevent evaporation. Sodium and potassium were analyzed with a Baird flamephotometer using internal standards, and chlorides by the method of Schales and Schales(2). Serum, renal lymph, and thoracic duct lymph proteins were analyzed with a Spinco paper electrophoresis apparatus while total protein was determined by the falling drop method(3). Heparin prevented clotting in blood and thoracic duct lymph samples.

Results. Concentrations of Na, Cl, and K in plasma, thoracic duct lymph and renal lymph are given in Table I. Na concentrations of plasma and thoracic duct lymph are similar (averages = 145.7 ± .77 and 145.6 ± 2.6 meq/l. Renal lymph, however, has an average concentration of 162.1 ± 2.8 meq/l, 11.3% higher than the average plasma value, a difference which is significant (P value = <.001). The average value for thoracic duct lymph Cl of 121.3 ± 2.5 meq/l is 9.8% higher than average plasma Cl value of 110.5 ± 4.3 meq/l. This difference is not statistically significant. However, the average Cl concentration of renal lymph is 140.2 ± 3.8 meq/l, 26.9% higher than the average plasma Cl concentration; a significant difference (P value = <.001). There is no apparent correlation between lymph flows and Na concentration as judged by data from 14 dogs or Cl concentration from 6 dogs. The mean K values for all 3 fluids are similar: 4.03, 3.95, and

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* Supported by grant from Medical Research and Development Division, Office of Surgeon General, Dept. of Army.

TABLE I. Control Values (meq/l) for Renal Lymph, Plasma, and Thoracic Duct Lymph, in 30 Dogs.

Lymph flow, ml/min.	Sodium R.L.	Sodium P.	Sodium T.D.L.	Chloride R.L.	Chloride P.	Chloride T.D.L.	Potassium R.L.	Potassium P.	Potassium T.D.L.
	151.6	140.0		125.6	99.3		4.0	4.0	
	190.0	148.4		159.3	100.8				
	153.2	145.2		126.2	84.5		3.6	3.2	
	206.2	146.8	155.2	152.0	132.0	106.5			
	171.0	151.6	146.8	144.0	122.0	115.5			
	164.0	148.4	148.4	152.0	122.0	132.0			
	180.0	151.4	152.8				3.5	3.7	4.4
	172.5	144.3	152.0	164.1	119.5	126.2	3.9	4.5	4.1
		144.0	153.3		80.6	120.9	3.8	4.0	4.4
				151.4	80.6	124.2			
.025	155.0	139.0	146.0	138.2	119.1	122.2	3.9	4.1	3.9
	158.0	140.9	116.6				4.1	4.0	3.5
.015	167.5	146.1	147.3						
	153.5	143.7	139.5						
	169.3	141.3							
	161.3	147.1	152.1	129.7	119.7	123.1			
	152.9	137.7	127.4						
	156.6	140.0	145.9						
	167.3	142.6	148.5				4.2	3.8	3.9
.003	164.3	139.5							
.007	157.3	145.8							
.026	156.0	150.9	152.6				3.9	3.7	4.1
	126.3	144.8							
	149.3	142.4							
	171.7	159.7					5.5	4.8	
.001	150.0	147.5		147.0	125.5				
.04	147.3	148.9		124.6	110.1		4.3	4.1	
.002				133.7	124.2		3.6	3.5	
	164.8	153.2		114.4	118.0				
	160.0	148.5							
Avg	162.1	145.7	145.6	140.2	110.5	121.3	4.03	3.95	4.04

4.04 meq/l for renal lymph, plasma, and thoracic duct lymph, respectively.

Electrophoretic analyses of 5 samples indicate no significant differences between renal lymph and serum in relative proportions of albumin, alpha one, alpha two, beta and gamma globulins. Renal lymph concentration of total proteins is about 60% that of serum while thoracic duct concentration is about 70%. Fibrinogen is present in renal and thoracic duct lymph but the amounts have not been quantified.

Discussion. Our findings lend support to an hypothesis suggested by our data and that of others that renal lymph in the normal functioning kidney is derived from 2 sources: capillary filtrate and tubular reabsorbate. If renal lymph were derived solely from capillary filtrate, there should be no significant differences in electrolyte concentrations of renal lymph and plasma except perhaps that Cl might be slightly higher due to the Donnan effect. Smith(4) suggests that the distal tubule may be able to reabsorb solute independent of water. Thus, in the absence of antidiuretic hormone stimulation, distal tubule reabsorbate containing a concentrated solution of Na and Cl may be mixed with capillary filtrate to form renal lymph. Recent work of Wilde and Malvin(5) indicates the existence of a distal area of very active Na reabsorption. The distal tubule is further implicated as a source of renal lymph by the finding of lower glucose concentrations in renal lymph than in plasma(6) suggesting that renal lymph glucose is derived solely from capillary filtrate and its lower value is due to slight dilution by concentrated reabsorbate. Although we have no measure of total renal lymph flow, the small flows (0.001-0.04 ml/min) obtained from the single capsular lymphatic studied make it unlikely that renal lymphatics play a major role in transporting reabsorbate in the normal kidney.

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Fall in Uterine Histamine Associated with Ovum Implantation in Pregnant Rat.* (24634)

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A role for histamine as decidual cell inducer in the mechanism of ovum implantation has been proposed(1-4). Evidence includes: 1) induction of deciduomata (DCM) by intraluminal histamine(1,5). 2) inhibition of DCM by intraluminal histamine-antagonists (1), 3) induction of DCM by traces (30 $\mu\mu$) of intraluminal histamine releaser 48/80(6), 4) induction of DCM by systemic (i.p. and i.v.) injections of histamine and of histamine releasers(6), 5) inhibition of systemically induced DCM by intraluminal histamine antagonists(6), 6) inhbiition of natural ovum implantation by intraluminal antihistamine(2), 7) mast cell depletion of rat endometrium immediately before nidation, and 8) intense infiltration of eosinophilic polymorphs before nidation (unpublished). This report measures histamine of uteri of rats during period of ovum implantation.

Methods and materials. Forty-five multiparous female rats (200 ± 20 g) were mated and examined for vaginal sperm before 9 a.m. the next morning. At intervals of 96, 120, and 144 hr after detection of sperm, groups of 5 rats were killed and their uteri removed and weighed. Histamine was extracted in 10% TCA(7). Uteri of 5 rats were pooled for each time-sample. Bioassays (4-pt) were carried out on atropinized guinea pig gut. Confirmation of histamine was by use of histamine antagonist (tripelennamine) at termination of assay. Three groups were done, all under same conditions.

Results. Figure shows values obtained; histamine concentration (μg/g) and total content (μg). Histamine concentration and content of uteri were markedly reduced between 96 hr and 120-144 hr.

Discussion. Since ovum implantation occurs in rats of this colony around 120-135 hrs after mating, and sperm were detected about 8 hr following mating, reduction in histamine concentration and content of gravid uteri took place just prior to implantation. This correlates with the fact that mast cell population of endometrium is reduced to almost zero during 80th to 120th hr after mating. Moreover this time interval fits the period between provocation of progravid endometrium and appearance of first nests of decidual cells(8). These findings add evidence for a role of histamine in the mechanism of nidation related to induction of decidual cell reaction. The release of histamine is effected by estrogens. Limited and preliminary histamine assays of 10%TCA extracts of uteri of spayed rats, 90 and 180 min after injection of 20 μg of estradiol show relase of histamine by estrogen(9). Spaziani and Szego(10) have recently demonstrated significant decrease of uterine histamine following single injection of estradiol-

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17. Studies recently completed in our laboratory show estradiol, estrone and estriol equally effective on a molar basis in releasing histamine.

Summary. Bioassay of uteri of pregnant rats reveal a significant reduction of concentration and content of histamine during 24 hr prior to actual implantation of blastocyst.

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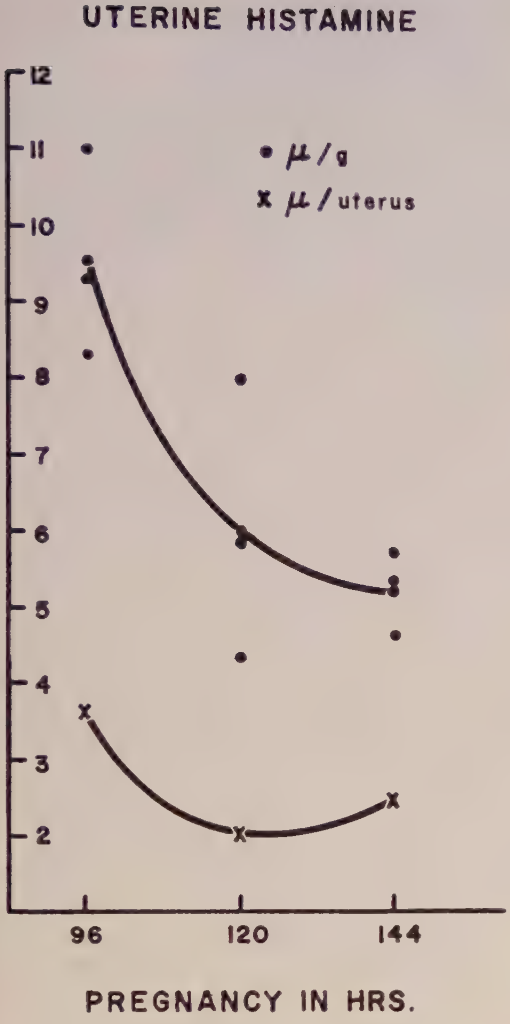

FIG. 1. Histamine concentration (μ/g) and content (μ/uterus) during early pregnancy. Curves connect means of 3 samples, each sample pool of 5 rats.

Detection of Depressant Components of Drug Activity. (24635)

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Seeking a rapid means of detecting subtle central depressant effects of drugs, we administered substances to mice followed by a subhypnotic dose of hexobarbital. In this, and in similar procedures(1,2,3), it is assumed that depressants will add to the effect of the barbiturate and induce loss of the righting reflex. To determine whether central depressant activity could be detected among drugs not ordinarily classified as CNS depressants, widely different types of compounds have been assayed with the results set forth below.

Method. Drugs were administered orally or intraperitoneally when necessary to unstarved, adult male albino mice (CF #1) in groups of 10, at 3 or more doses. Thirty minutes later, (or more, depending on time of peak activity of test drugs) a subhypnotic

TABLE I. Potentiation of Subhypnotic Dose of Hexobarbital.

Drug	ED_{50} (95% limits), mg/kg p.o.
Hydroxazine	250 (167– 375)
Meprobamate	123 (108– 140)
Prochlorperazine	21 (15– 30)
Chlorpromazine	4 (2– 6)
Phenaglycodol	62 (41– 94)
Reserpine*	5 (3– 8)
Perphenazine	7 (6– 8)
Thiopropazate	5 (3– 7)
Phenobarbital†	28 (18– 43)
Glutethimide	43 (33– 56)
Diphenylhydantoin*	23 (15– 36)
Trimethadione†	370 (255– 537)
Acetylsalicylic acid	900 (643–1260)
Morphine	14 (10– 19)
S.K.F. 525-A	No effect
Nikethamide	*Idem*
Ethopropazine	73 (50– 106)
Sulfanilamide	160 (103– 248)
Iproniazid	185 (137– 250)
Diphenhydramine	200 (149– 238)
Tripelennamine	Marked excitation

* Given 3 hr before hexobarbital.
† " 2 " " " .

dose of hexobarbital sodium (50 mg/kg) was injected intraperitoneally. Animals were placed on their backs and those unable to right within 2 minutes were counted. The dose required to induce loss of the righting reflex in 50% of the animals (ED_{50} with 95% fiducial limits) was computed by the method of Litchfield and Wilcoxon(4) with results summarized in Tables I and II. In addition, the time interval between loss and spontaneous return of the righting reflex was recorded in minutes for each dose. The interval corresponding to the ED_{50} was then estimated graphically. These results are not recorded.

Results. The first 14 compounds listed in Table I, generally regarded as central depressants, include analgesics, ataraxics, anticonvulsants, and sedative-hypnotics. Their effects in this test procedure involve, essentially, conversion of a subhypnotic dose of hexobarbital to a hypnotic one. Thus, at ED_{50} dose levels their effectiveness may be equated roughly to 20 mg/kg of hexobarbital intraperitoneally. The method is sufficiently sensitive to detect mild depression elicited by analgesics and ataraxics. More potent depressants are detected at small fractions of dose required to produce overt signs of depression. The ED_{50}'s, being inversely proportional to potency, provide a relative measure of CNS-depressant activity.

The next compound, β-diethylaminoethyldiphenylpropyl acetate (S.K.F. 525-A), gave negative results, although it prolongs the action of a hypnotic dose of hexobarbital(5) by inhibiting enzymes which oxidize the sidechain of hexobarbital(6). A similar mechanism cannot obtain in the present test because a hypnotic dose of the barbiturate is not given. Nikethamide prolongs the effect of a full hypnotic dose of hexobarbital in mice (unpublished observation) but, in agreement with its classification as a central stimulant, it was completely inactive in our test.

The remaining drugs listed in Tables I and II are not classed as CNS-depressants, yet loss of the righting reflex was induced by all of them, and in some instances (Compounds 17, 18, 19 and 20) dose-response relationship was linear. The antiparkinson drug (Ethopropazine) and sulfanilamide, both of which produce drowsiness in patients, potentiated the action of hexobarbital. Iproniazid induces convulsions and is used clinically to counteract depression. It inhibits monoamine oxidase which inactivates norepinephrine and serotonin in the brain(7), and this action, too, tends to support its classification as a stimulant. Nevertheless, in our study iproniazid exhibits depressant effects in the mouse. The antihistamines diphenhydramine and tripelen-

TABLE II. Non-Linear Potentiation of Subhypnotic Dose of Hexobarbital.

Drug	Dose and route of admin.	Loss of righting reflex (%)
Acetazolamide*	50 mg/kg, p.o.	20
	100	20
	200	40
	400	40
Epinephrine	.04 i.p.	20
	.08	20
Insulin	.05 U, i.p.	50
Atropine	150 mg/kg, p.o.	20
	200	60
	400	10
Adiphenine	200	30
	400	20
Homatropine methyl bromide	200	30
	400	30
	800	20

* Given 6 hr before hexobarbital.

namine are markedly excitant for mice, yet within a selected dose range diphenhydramine potentiated hexobarbital while tripelennamine was totally inactive. However, both drugs significantly prolong the effect of a full hypnotic dose of barbiturate(8).

Recording duration of loss of righting reflex is a time consuming procedure, but it discloses unusual effects. For example, with all drugs the interval averaged 30 (range 10 to 50) minutes, except with diphenhydramine which prolonged the duration beyond 3 hours. The mechanism of this reaction has not been investigated.

The 6 agents listed in Table II induced loss of the righting reflex, but dose-response relationship was not linear. Acetazoleamide, a carbonic anhydrase inhibitor, is effective in blocking epileptic seizures. Koch and Woodbury(9) reported that this compound decreases brain excitability, and concluded that its anticonvulsant properties stem from an increased concentration of carbonic acid within brain cells. This type of depression is readily detected. Epinephrine and other phenethylamines are known to cause some central depression. Thus, Quastel and Wheatley(10) suggested that epinephrine retards brain metabolism, and Ivy *et al.*(11) reported that it produced analgesia in man and animals. Later the hormone was shown to prolong the action of hypnotic doses of hexobarbital(12) and chloral hydrate(13). In agreement with earlier work, our experiments reveal a component of depressant activity. Insulin has been reported to depress brain metabolism (14) and to prolong the effect of an hypnotic dose of hexobarbital in mice(12). Similar results were obtained in the present test. Higher doses produce convulsions. The last 3 drugs listed in Table II are antispasmodics. They are capable of producing convulsions and are recognized central excitants. Nevertheless, they potentiated hexobarbital. Thus, it appears that these drugs possess depressant properties. The number of animals losing the righting reflex, however, decreased with increasing dosage, presumably because stimulation supervenes at higher doses.

Discussion. In our test, loss of the righting reflex is equated to central depression. No opinion can be offered as to mechanisms whereby this depression is brought about. The operator will have to discriminate between central depressants and drugs like succinylcholine which act peripherally. Distal effects which reduce the number of impulses reaching the sensorium and prepare the animal for sleep will be more subtle and more difficult to assess.

Summary. A method based upon potentiation of a subhypnotic dose of hexobarbital has been employed to detect depressant activity of drugs. It is rapid, and gives reproducible results with known CNS depressants. It appears to differentiate depressant drugs from those which prolong the action of a hypnotic dose of hexobarbital by interference with biotransformation mechanisms. It affords a rapid means of detecting depressant activity of drugs not ordinarily classified as central depressants.

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Continuous Cultivation of Malignant Cells from a Canine Testicular Tumor.
(24636)

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This paper describes development in tissue culture of a continuous line of malignant cells from a canine Sertoli cell tumor (SCT) and reports susceptibility of these cells to different viruses.

Materials and methods. The Small Animal Surgery Clinic, School of Veterinary Medicine, Univ. of Pa., received a 13-year-old, obese, male West Highland terrier. The owners had noted a mass in the scrotum about one month previously. Physical examination revealed enlarged mammary glands with hyperpigmented nipples, symmetrical alopecia of inguinal, abdominal and thoracic areas, hyperpigmentation about the anus, scrotum and abdomen. The right testis was firm and greatly enlarged while the left was atrophic. After castration, specimens of testis were retained for study.* Histopathological examination revealed a Sertoli cell adenocarcinoma.† Tumor material was removed aseptically from the surgical specimen and immediately placed in screw cap centrifuge tubes containing 20-30 ml Hanks' Balanced Salt Solution (BSS) and Penicillin, 100 units/ml and Streptomycin 100 μg/ml. After 2 washings in fresh BSS containing antibiotics, approximately 5 mm^3 was removed from center of tumor and placed in 10% formalin. Remaining tissue was cut into 1-2 mm pieces and transferred to sterile 500 ml Erlenmeyer flask containing sterile magnet and 100 ml of 0.5% trypsin. An electrically heated jacket to maintain constant temperature of 37°C was secured around the flask and the entire assembly then placed on magnetic stirrer and agitated 25 minutes. The cellular suspension was separated aseptically from tissue fragments by straining through 2 layers of sterile cheesecloth. Fresh trypsin was then added to flask and this process repeated at 25 minute intervals until practically all tissue was disintegrated. Strained suspensions were stored in ice bath, then centrifuged in the cold at 600 rpm for 30 minutes. The cells were then seeded into conventional screw-cap milk-dilution bottles after cellular suspension had been adjusted to contain one million cells/ml. The count was made in standard haemocytometer counting chamber. Three media were used for growth of these cells—medium 199 plus 10% inactivated horse serum, Basal Medium Eagle (BME) plus 10% inactivated horse serum and Enders' Medium (1). Each medium contained 100 units Penicillin, 100 μg Streptomycin and 50 units Mycostatin/ml. Incubation was at 37°C. Growth media were replaced with fresh media at 3-day intervals. After 3-4 days incubation a fibroblast-like cell appeared and predominated in all cultures. Within 1 to 2 weeks most of these spindle-like cells separated, enlarged greatly and disintegrated. Continued "blind" feeding with fresh growth media for 3 to 4 more weeks resulted in appearance of a few clumps of epithelial-like cells in several bottles. These cells grew fairly rapidly and soon became visible macroscopically. At this time, the cells in one bottle with approximately one-third of its surface covered with isolated clumps of cells, were trypsinized and a new milk-dilution bottle inoculated. This redistribution of cells throughout entire bottle resulted in even seeding. Serial passages carried out by adjusting cellular suspensions of the seeding material to contain 100,000 cells/ml resulted in appearance of full monolayers of cells in 3-4 days. A cytological record of stained cells were kept after each trypsinization step. Coverslips in Leighton tubes were seeded with 1 ml of suspension and allowed to grow for 3-4 days. Coverslips were then fixed in methyl alcohol for 15 minutes and stained with Giemsa. At time of this report the cell line has undergone 55 passages.

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* I am indebted to Dr. R. Brodey, School of Vet. Med., Univ. of Pennsylvania, for obtaining tumor material.

† Acknowledgement is gratefully given to Dr. J. McGrath, School of Vet. Med., Univ. of Pennsylvania, for histological study of this tumor.

TABLE I. Effect of Maintenance Media and Storage for 2 Months on Survival of SCT Cells *In Vitro.*

Medium No.	Composition	%	Storage temp., °C	Survival*
1	Balanced salt sol.	37.5	4	0
	Bovine amniotic fluid	37.5		
	Horse serum	20	37	0
	Bovine embryo extr.	5		
2	Medium 199	98	"	4+
	Horse serum	2		
3	Medium 199	98	4	3+
	Calf serum	2	37	4+
4	Medium 199	100	"	2+
5	Basal medium, Eagle	98	"	0
	Calf serum	2		
6	Basal medium, Eagle	98	"	0
	Horse serum	2		
7	Balanced salt sol.	95	"	0
	Horse serum	5		
8	Basal medium, Eagle	90	4	3+
	Horse serum	10	R.T.†	1+
9	Medium 199	90	4	3+
	Horse serum	10	R.T.	3+

* Survival was determined by sampling aliquots of cells after 2 mo storage at various temperatures. Appearance of viable cells and degree of growth obtained in tubes or bottles after reincubation for 24 hr and then refeeding with fresh growth media was graded. 0 indicates no growth; 1+ to 3+, intermediate degrees of growth, and 4+, a confluent sheet of cells. All bottles incubated at least 7 days at 37°C.

† Room temperature.

Results. Cells (SCT) were cultivated on glass surface of roller tubes. The citric acid-crystal violet technic was used for determination of viable cells. The media studied were: medium 199 + 10% horse serum, BME + 10% horse serum and Enders' medium. Although growth rate in Blake bottles with medium 199 was fairly similar to growth rate in BME, appearance of cells in each medium was different. In BME the cells tended to grow in discrete patches while in medium 199 the cells were evenly distributed. There was no appreciable growth in Enders' medium.

Table I shows results of these experiments as well as effects of storage. Of media tried only those incorporating medium 199 with small amounts of serum had any beneficial effect in maintaining the cells in storage. Attempts to store the cells at –70°C were unsuccessful.

Animal inoculation. Since an important criterion in the definition of malignancy is establishment of a tumor in a susceptible host (2), varying numbers of SCT cells (250 thousand - 12.5 million) suspended in medium 199 were inoculated into mice, rabbits, guinea pigs, and embryonated eggs. These hosts were refractory to the tumor. SCT cells of 7th and 10th tissue culture passage were inoculated into cheek pouches of Syrian hamsters, conditioned according to technics described by Toolan(3), and Lutz, *et al.*(4), with negative results, until cells of 24th tissue culture passage were used. At this passage level, 9 out of 12 hamsters developed tumors approximately 0.2 cm^3, within 5 days. However, one week after inoculation tumors in all animals except one regressed. Attempts serially to pass this remaining tumor in the hamster as well as in tissue culture failed. These negative results prompted us to investigate the possibility of a tumor response in the homologous host. Accordingly, three, 3-4 month old, male mongrel dogs were inoculated with trypsinized, washed SCT cells of the 10th passage. As controls, male dogs, 3-4 months old were inoculated with diluent. Immediately following inoculation, all dogs were treated with metacortelone acetate (Schering) 75 mg/dog for the first 5 days, and 20 mg/dog for the next 5 days.

The results (Table II) show that tumors were obtained in 2 out of 3 dogs inoculated

TABLE II. Effect of Tissue Culture Propagated SCT Cells on Tumor Formation in Dogs.

Dog No.	Route*	No. SCT cells inj./dog ($\times 10^6$)	Tumor formation and size	
1	IP	50	Neg.	
	IM	20	Pos.	10 × 20 mm
	IT	2.5	Neg.	
	AE	1	"	
2	IP	50	Pos.	Numerous masses 5 × 10 mm in size
	IM	20	"	40 × 20 × 10 mm
	IT	2.5	Neg.	
	AE	1	"	
3	IP	50	"	
	IM	20	"	
	IT	2.5	"	
	AE	1	"	
4 & 5	IP, IM, IT, AE	None	"	

* IP, intraperitoneal; IM, intramuscular; IT, intratesticular; AE, anterior chamber of eye.

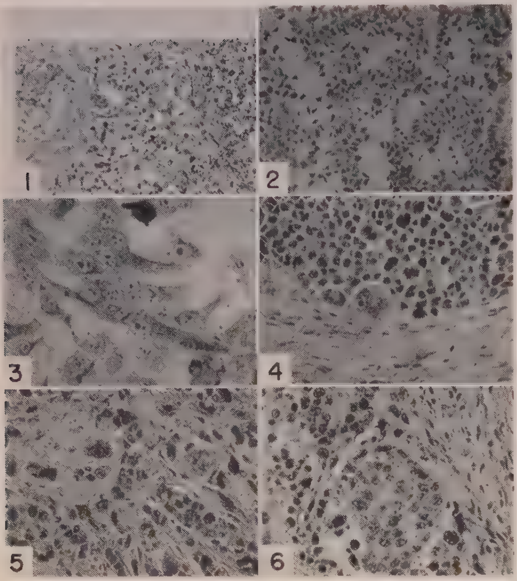

FIG. 1. Histological section of original dog tumor (H & E × 72).

FIG. 2. Forty-eight hour culture of SCT cells of 10th tissue culture passage (Giemsa × 72).

FIG. 3. Same (Giemsa × 288).

FIG. 4. Histological section of intramusc. tumor removed from dog #1 inj. intramusc. with SCT cells of 10th tissue culture passage (H & E × 180).

FIG. 5. Same from dog #2 inj. intraper. with SCT cells of 10th tissue culture passage (H & E × 180).

FIG. 6. Section of intramusc. tumor from dog #2 previously inoculated intramusc. with SCT cells of 10th tissue culture passage (H & E × 180).

with 10th tissue culture passage SCT cells. Histological and pathological examination of H & E- and Giemsa-stained preparations from these tumors revealed nodular neoplastic tissue arranged in cords and nests separated by loose fibrous tissue. Tumor material derived from intramuscular inoculation revealed tumor cells varying greatly in size, shape, nuclear pattern and affinity for hematoxylin. Many multinucleated tumor giant cells and giant nuclei were present. In the less anaplastic areas the cells retained some similarity to Sertoli cells. Examination of control material from each control dog at each site of inoculation revealed no such change of appearance of normal cells.[‡]

Fig. 1-6 show histopathological and cytological characteristics of this tumor at time of castration, at 10th tissue culture passage and after removal of tumors from 2 dogs inoculated with 10th passage SCT cells.

Tumor cell susceptibility to viruses. A number of viruses have been tested for their capacity to produce cytopathogenic changes in 40th tissue culture passage of this cell line. One tenth ml of 10^{-1}, 10^{-3} and 10^{-5} dilution of each virus was inoculated into SCT cells. Infected cultures were observed daily for 7 days for appearance of maximal cytopathogenic effects. Cultures designated negative, represent 3 successive passages in SCT cells. Table III shows the viral spectrum of this SCT line.

Cytological changes in SCT cells were generally observed 2 to 7 days after inoculation of roller tubes with serial 10-fold dilutions of each test virus. Control cells were invariably

TABLE III. SCT Cells Susceptibility to Viruses.

Virus	Type or strain	Virus source	Cytopathogenic effects
Newcastle	"B"	Chick allantoic fluid	+
Semliki Forest		10% mouse brain	+
Vaccinia		Bovine embryonic skin TC	+
Herpes simplex		Rabbit kidney TC	+
Canine hepatitis		Canine kidney TC	+
Influenza	Jap. 305	Chick allantoic fluid	—
	Formosa	*Idem*	—
	PR-8	”	—
Adenovirus	7	Monkey kidney TC	—
Measles	Edmonston	*Idem*	—
	”	Human amnion TC	—
Echo	6	Monkey kidney TC	—
	9	*Idem*	—
Coxsackie	B	”	—
Rabies		10% mouse brain	—
Canine distemper		Commercial chick embryo vaccine	—
Rous sarcoma		Chick embryo CAM	—

[‡] Acknowledgement is gratefully given to G. Keller and Dr. W. Beckfield, of our Inst. for histological examination of these tumors.

polygonal but occasionally round cells were observed. Upon staining, these latter cells were in the process of division. Action of vaccinia virus was characterized by rounding of the cells 48 hours after inoculation, followed on 3rd or 4th day by noticeable swelling and distention of cytoplasmic elements of cells. By the 5th day cells lysed. With herpes simplex, semliki forest and newcastle disease viruses, distinct foci of degeneration first appeared within 64 hours then gradually progressed over entire culture in 148 hours. Infection with canine hepatitis virus was not evident until 5-7 days after inoculation. At this time, cell damage was characterized by appearance of numerous masses of round cells, packets of clumps of cells and eventual destruction of most cells 3-5 days after first signs of infection.

Summary. Growth characteristics of a canine Sertoli cell adenocarcinoma in tissue culture are described. Histopathological and cytological picture of the original tumor, of cells grown in tissue culture and material removed from dogs inoculated with tissue culture cells are shown. According to histopathological criteria, the original tumor, tissue culture cells and material removed from dogs inoculated with 10th passage SCT cells are all malignant. The viral spectrum of this cell line is presented.

Technical assistance of Marlene Ronchetti is gratefully acknowledged.

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Effect of Continuous Low Dose Gamma Irradiation on Bactericidal Activity of Phagocytic Cells of Mice.* (24637)

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Previous studies(1,2) on bactericidal activity of phagocytic cells of mice indicated that this function was impaired in cells obtained from mice which had been subjected to whole body x-radiation in a single acute exposure within the range of 500-700 r. It was of interest, therefore, to determine whether or not bactericidal activity would be similarly impaired by continuous exposure to relatively low doses of γ irradiation.

Materials and methods. Procedures and reagents for studying bactericidal activity of phagocytic cells from mice (Carworth, CF-1) were essentially those previously described (1). Peritoneal exudates were induced in mice with gelatin. Sixteen hours later, *Pseudomonas aeruginosa* organisms were injected into peritoneal cavities so that phagocytosis could occur *in vivo*. Antibiotics were then injected to kill extracellular organisms and the phagocytes were harvested, pooled, washed, and suspended (10^6 cells/ml) in tissue culture medium with antibiotics. Immediately, and at 2 and 4 hours thereafter, aliquots of the phagocyte suspension were ground and cultured to determine number of viable bacteria/ml. A total of 150 mice were housed in groups of 10 in plastic cages (7½ x 12 x 7 inches) surrounding a nominal 10 curie cobalt 60 source. The cages were placed on curved wooden racks so that their centers were 127

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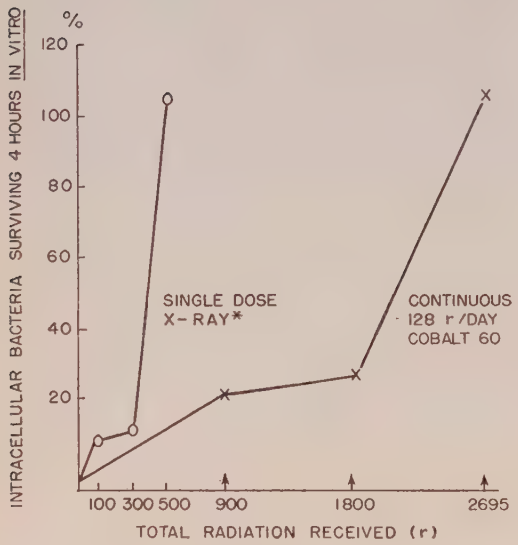
TABLE I. Effect of Continuous Gamma Irradiation (128 r/d) on Bactericidal Activity and Number of Exudate Cells in Mice.

Animal group Irradiation				Mean No. viable *Pseudomonas* $\times 10^4$/ml phagocytes			
Time, wk	Accumulated dose, r	Phagocytes per mouse, mm^3	% viable* phagocytes after 4 hr *in vitro*	Hr *in vitro* 0	2	4	4 hr count as % of 0 hr count
0	0	3.4	97	50.0	9.0	1.0	2%
1	900	3.3	92	5.3	3.5	1.2	21
2	1800	1.4	92	56.5	35.7	15.3	27
3	2695	1.2	87	38.5	21.5	55.8	105

* Cells with nucleus unstained by trypan blue (.0005%).



*BASED ON PREVIOUSLY PUBLISHED DATA (1)

FIG. 1.

cm from the source.‡ Under these conditions and assuming random movement of the mice, each mouse received approximately 128 r/22 hours (the source was shielded 2 hours a day to allow for cleaning cages and feeding the animals). Groups of 30 mice were removed from the cobalt room after 1, 2, and 3 weeks exposure, and their phagocytes examined for bactericidal activity. A group of 30 mice, similarly housed but not exposed to irradiation, served as controls.

Results. A summary of the results is presented in Table I. As previously shown(1), the number of exudate cells which could be recovered from the mice decreased with increasing amounts of irradiation. Extent of decrease, however, was not as marked as when the same amount of x-radiation was given in a single dose.

The volume of cells (1.2 mm^3/mouse) obtained from mice which had accumulated 2695 r during 3 weeks exposure at 128 r/d was about the same as that obtained from mice irradiated with 500 r in a single acute exposure.

The decrease in bactericidal activity was also less marked when the mice were exposed to 128 r/day. A substantial fall in bactericidal activity occurred only after a total of 1800 r had been administered, whereas 500 r was sufficient to cause a similar decrease when administered in a single dose. Fig. 1 illustrates the differences between effects of single dose x-irradiation and continuous low dose gamma irradiation on bactericidal activity. The percentage of those intracellular bacteria initially present which were still viable after 4 hours *in vitro* is plotted against total amount of irradiation administered.

Summary. Mice were subjected to continuous gamma radiation (128 r/22 hours) for 3 weeks, and bactericidal activity of their phagocytes determined at weekly intervals. Bactericidal activity declined gradually through 2 weeks exposure (1800 r) then fell off rapidly in the third week (2695 r). These results are compared to those obtained with mice exposed to single doses of x-irradiation.

1. Nelson, E. L., Becker, J. R., *J. Infect. Dis.*, 1959, v104, 13.

2. ———, *ibid.*, 1959, v104, 20.

Received November 20, 1958. P.S.E.B.M., 1959, v100.

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‡ Dosimetry and planning for irradiation of animals were carried out by members of research staff of Argonne Cancer Research Hosp., USAEC, at Univ. of Chicago.

Some Properties of Hypotensive Smooth Muscle Contracting Principle in Placenta of Rabbit.* (24638)

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In experiments on placental transfer of insulin in the rabbit(1) damage to the placenta was frequently followed by rise in maternal blood sugar and development of a shock-like state ending in death. An attempt was made to investigate the hyperglycemic phenomenon by preparation and intravenous injection of saline extracts of the placenta. No consistent effect on blood sugar of adult rabbits was noted. However, extracts proved to be very toxic, producing gasping respiration, marked intestinal and uterine peristalsis, sustained fall in blood pressure, and death, apparently from respiratory failure. On opening the chest after death, the lungs were pale and bloodless, the heart grossly dilated, especially the right chambers, and ventricular fibrillation was frequently observed. These effects resembled those of histamine and serotonin. A preliminary characterization of the active principle of rabbit placental extracts has been carried out. It has been shown that most activity is found in the fetal part of the placenta and that it is probably due to a polypeptide.

Methods. Investigation of some physical and chemical properties of the active extract showed that it was stable to heat at a neutral pH for 3 minutes at 100°C. Longer periods of heat at neutral pH were not tried. The extract was unstable to alkali and acid (1 hour at 100°C in 0.2 N HCl or NaOH), completely dialysable with about half the activity appearing in the dialysate in 24 hours when there were equal volumes inside and outside the sac. All activity was lost on ashing at 600°C for 24 hours. Standardization of extraction procedure was then established as follows: fetal placentae were separated from decidual tissues, weighed, and placed in graduated cylinder. Distilled water was added to make final volume 3 times that of the wet placentae and the whole homogenized in Waring blendor. The crude extract was then boiled for about 2 minutes. Each cubic centimeter of crude extract was equivalent to 0.33 g of wet placenta. The boiled extract was then placed in dialysis sac and dialysed against an equal volume of distilled water for 24 hours. Each cubic centimeter of dialysate was equal to about 0.166 g of wet placenta.

Results. The dialysate was assayed on an isolated loop of guinea pig ileum in an oxygenated bath (20 ml) of Tyrode's solution. Two-tenths ml of the dialysate (equivalent to 0.0332 g of wet placenta) caused a sustained contraction. The type of contraction produced by this amount of extract was slower and more sustained than that produced by histamine; also, the extract was washed out from the tissue with much greater difficulty. Contraction produced by the extract was not blocked by an amount of atropine (1 μg/ml, final concentration in bath) sufficient to inhibit the effect of acetylcholine (0.05 μg/ml). Similarly, the contraction was not blocked by an amount of benadryl (0.5 μg/ml) or pyribenzamine (1.0 μg/ml) sufficient to inhibit the effect of histamine (0.05 μg/ml), nor by an amount of lysergic acid diethylamide (1.0 μg/ml, enough to inhibit the effect of serotonin (0.05 μg/ml).

When tested in anesthetized rabbits (3 to 4 kg) and a dog (10 kg), the dialysate caused a fall in blood pressure after intravenous injection. One ml of extract caused a fall of about 60 mm of mercury in the rabbit and 8 ml caused a fall of about the same amount in the dog. There was a tendency for the blood pressure to remain at shock levels in the rabbit after a single injection but not in the dog. Extracts of other tissues (liver, lung, uterus, and maternal placenta) from adult rabbits extracted under identical conditions and equivalent in wet weight to the placental extract, were found to be without effect on the guinea pig ileum and on blood pressure of rabbit and dog. The hypotensive action of placental extract in the rabbit was easily differen-

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* Supported by grant from Josiah Macy, Jr., Fn.

tiated from that of histamine, serotonin, and acetylcholine by use of the aforementioned antagonists.

Further purification of the dialysate from placental extracts was carried out by paper chromatography. Ten to 30 ml of the dialysate were evaporated to dryness under vacuum and the residue taken up in a small volume of distilled water. The latter was then placed on strip of Whatman paper No. 1 and run as a descending chromatogram using butanol saturated with 10% ammonium hydroxide as solvent. After 20 hours the strip was dried and cut into horizontal strips 2 cm wide beginning at origin and extending to solvent front. Each strip was then placed in a test tube and eluted with 2 ml of Tyrode's solution. The contents of each tube were then assayed on guinea pig ileum. A rather precise separation of the active placental principle was accomplished consistently by this procedure and was regularly found in the same eluates after 20 hours on the chromatogram paper. Two-tenths ml was active in causing contraction of the ileum and, as before, the effect was not blocked by benadryl and pyribenzamine nor by atropine and lysergic acid diethylamide. The eluates which were active on guinea pig ileum were also effective in lowering blood pressure of rabbit. These results suggest that the hypotensive placental factor and the guinea pig ileum-contracting factor are identical.

Paper chromatograms were developed in ultra-violet light at 260 mμ on Kodak contact standard (specification 87) photographic paper. No absorbing material was found in areas associated with the action on guinea pig ileum, indicating that the principle does not contain nucleotides or other ultra-violet absorbing materials. When histamine and the dialysate from the placental extract were tested on the same chromatogram there was separation but the separation was not sharp. The Rf value of the dialysate ranged from .38 to .54 while that of histamine was constant at .54. Serotonin had an Rf of .62 and there was complete separation from the active principle of the placental extract. Extracts were subjected to paper chromatography and spectrophotofluorometric analysis by the method of Udenfriend, Weissbach, and Clark (2). Both methods proved negative for serotonin. Histamine could not be identified on the chromatograms using the method of Urbach(3). The absence of free reducing groups was shown when chromatograms were sprayed with benzidine reagent with negative results.

Paper chromatograms of dialysate from the placental extract were sprayed with 0.125% ninhydrin in acetone and heated 5 minutes at 100°C. No ninhydrin-reacting material was observed in the area of the chromatogram associated with activity on guinea pig ileum, showing that the activity was not related to presence of free amino acids. Eluates containing active materials from paper chromatograms of plancental extract were made with 4 ml of distilled water and divided into 2 portions. One portion was hydrolysed with 6 N hydrochloric acid for 48 hours at 100°C; the other was stored at 4°C without acid. Both aliquots were then tested on paper chromatograms, as described above, and after control analyses on guinea pig ileum, were sprayed with ninhydrin (0.125% in acetone), and heated 5 minutes at 100°C. There was no contraction of guinea pig ileum caused by the hydrolysed portion of the chromatogram but abundant ninhydrin color was present. The unhydrolysed portion of the chromatogram was still active and no ninhydrin color was present in the acvtive area. These results show that amino acids are liberated on hydrolysis of the active principle.

No significant change in activity of placental extracts was observed from 16th to 27th day of pregnancy in the rabbit. A dialysable, heat stable principle, active on rabbit blood pressure and guinea pig ileum was observed in extracts of the placenta of cow, guinea pig, and pre-eclamptic human patient. The contractile response of the ileum with cow and guinea pig placental extracts was of the rapid type, however, and more closely resembled the effect of histamine, while that of human was of slow and sustained type. Further characterization of the active principle of these placentae has not been carried out.

On evidence presented above it seems possible to rule out with certainty all of known compounds with activities similar to those of rabbit fetal placental extracts, namely serotonin, histamine, acetylcholine, nucleotides, and inorganic ions. The evidence points to a substance of low molecular weight and probably a polypeptide similar to known polypeptides which are active on smooth muscle such as bradykinin, kallidin, and substance P(4), The presence of this powerful substance in fetal placenta of rabbit poses a problem in relation to the physiology of gestation, a matter of conjecture at present. Further studies on the substance, especially in relation to its activity on the uterus, are planned.

Summary. 1) A hypotensive, smooth muscle-contracting principle has been identified in the placenta of the rabbit and of several other species. It is dialysable, stable to heat at neutral pH for 3 minutes at 100°C, and unstable in alkali and acid. It has been separated chromatographically and by use of inhibitors from acetyl choline, serotonin, and histamine. The active principle is hydrolysable with liberation of free amino acids and with resulting loss of activity on guinea pig ileum and blood pressure. It has been tentatively identified as a polypeptide. 2) On intravenous injection the active principle causes a fall in blood pressure, cardiac dilatation, intestinal and uterine peristalsis, and death from respiratory failure. It is found predominantly in the fetal part of the placenta and at all stages of pregnancy in the rabbit from 16th day to term.

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2. Udenfriend, S., Weissbach, H., Clark, C. T., *J. Biol. Chem.*, 1955, v215, 337.

3. Urbach, K. F., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 146.

4. Gaddum, J. H. (ed.), *Polypeptides Which Stimulate Plain Muscle,* E. and S. Livingstone, London, 1955.

Received November 20, 1958. P.S.E.B.M., 1959, v100.

Influence of Prochlorperazine on Hydrocortisone Inhibition of *in vitro* Leucocyte Migration.* (24639)

MELVIN M. KETCHEL AND SOMERS H. STURGIS (Introduced by L. K. Diamond)
(With technical assistance of Aurelia C. Koros and Nancy S. Crowell)
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Addition of hydrocortisone to human blood has been shown by Ketchel, Favour, and Sturgis(1), to inhibit amoeboid migration of leucocytes in capillary tubes. Several lines of reasoning suggest that hydrocortisone was acting in its capacity as a hormone, rather than in non-specific manner. These reasons are, (1) similar chemical structures, such as tetrahydrocortisone and desoxycorticosterone, were not effective; (2) hydrocortisone was effective in concentrations similar to those which occur in blood; and (3) hydrocortisone from several manufacturers, including both alcohol-free hydrocortisone and hydrocortisone sodium succinate, were effective, thus decreasing the possibility that a toxic contaminant of the hydrocortisone could be responsible for the effect. During these experiments, it was noted that leucocytes of a patient being treated with prochlorperazine[†] were unaffected by concentrations of hydrocortisone, which had been effective before the patient was given prochlorperazine. This chance observation led to *in vitro* experiments, here described, which attempted to clarify the effect of prochlorperazine on response of leucocytes to hydrocortisone.

Methods and materials. In the capillary tube method of studying leucocyte migration (Ketchel and Favour(2)), small bore capillary tubes are partially filled with blood, and end

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* Work done under grants from N.I.H., U.S.P.H.S.

[†] Compazine, Smith Kline and French Co., Philadelphia, Pa.

TABLE I. Sample Protocol for Combining Leucocytes with Hydrocortisone and Prochlorperazine.

Mixing vial No.	Cells	Plasma	Final conc. (μg/ml) of Prochlorperazine	Hydrocortisone	Hanks' sol.
	ml	ml	(.1 ml added)	(.1 ml added)	
1	.2	.3	100	100	
2	"	"	10	"	
3	"	"	1	"	
4	"	"	.1	"	
5	"	"	100		.1
6	"	"	10		"
7	"	"	1		"
8	"	"	.1		"
9	"	"		100	"
10	"	"			.2
11	"	"			"

Mixing vials 1 through 4 received both hydrocortisone and prochlorperazine. Mixing vials 5 through 8 received prochlorperazine alone. Mixing vial 9 received hydrocortisone alone; mixing vials 10 and 11 received no hydrocortisone and no prochlorperazine.

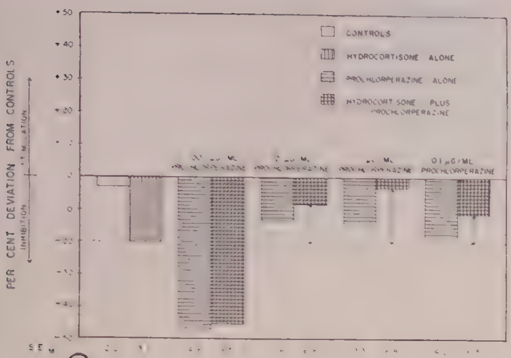
of each tube sealed. Centrifugation results in a system with red cells at bottom of capillary tube, a "buffy coat" of leucocytes over the red cells, and clear plasma at the top. When the capillary tube is incubated vertically, leucocytes move by active amoeboid motion through interstices of clotted plasma or along the walls of capillary tube. The distance which leucocytes move away from the original buffy coat in a standard incubation period, is measured with ocular micrometer in microscope. Details of preparing capillary tubes, were the same as described in earlier papers: Ketchel and Favour(2); Ketchel, Favour, and Sturgis (1). Hydrocortisone sodium succinate[‡] and prochlorperazine were dissolved in Hanks' Solution (Hanks and Wallace(3)), and dilutions made so that addition of 0.1 ml to cell suspension would make the desired final concentration. Hanks' solution without added hydrocortisone or prochlorperazine was added when necessary to keep volume relationships constant (Table I). The same cell suspensions, plasma, and other reagents were used throughout each experiment, and the order of preparation of capillary tubes from the mixing vials was randomized. Ten capillary tubes were prepared from each mixing vial, and the results averaged. Mixing vial 10 was called the control, and average migration from capillary tubes prepared from it, was compared with average migration obtained from capillary tubes prepared from the other mixing vials. Inclusion of mixing vial 11, identical to mixing vial 10, provided information on variability of the method.

Results. The results of 11 experiments, to determine effects on leucocyte migration of prochlorperazine and hydrocortisone alone and in combination, are shown in Fig. 1. In this and following figures, results are indicated as average per cent deviation which occurred in capillary tubes made from experimental mixing vials, as compared to capillary tubes made from control mixing vials which contained no prochlorperazine or hydrocortisone. A series of capillary tubes from a second control mixing vial was included in each experiment to determine variation in experimental procedure, and average percent deviation which occurred in capillary tubes made from each second control mixing vial is indicated in the Figures as "controls." By including this second control, percent deviation of controls could be compared statistically to per cent deviation which occurred with hydrocortisone or prochlorperazine. Dotted line in Fig. 1 represents deviation which occurred when hydrocortisone alone was added to the cells, and arrows indicate change from hydrocortisone inhibition which occurred as each concentration of prochlorperazine was added to hydrocortisone in the tubes. In Fig. 1, average per cent deviation of second controls from the first was –3% ± 2.2. Average deviation caused by hydrocortisone was –20% ± 3.1. This inhibition caused by hydrocortisone is significant at the 0.01 level.

As further shown in Fig. 1, addition of 100 mg/ml of prochlorperazine to blood inhibited leucocyte migration by almost 50%, and this was neither increased nor decreased by addition of hydrocortisone. Addition of 10 μg/ml of prochlorperazine caused an average deviation from controls of –13% ± 2.2. The combination of 10 μg/ml of prochlorperazine and 100 μg/ml of hydrocortisone reduced average deviation to –7% ± 6.6.

The effect which occurred at concentration

[‡] Solu-Cortef, Upjohn Co., Kalamazoo, Mich.



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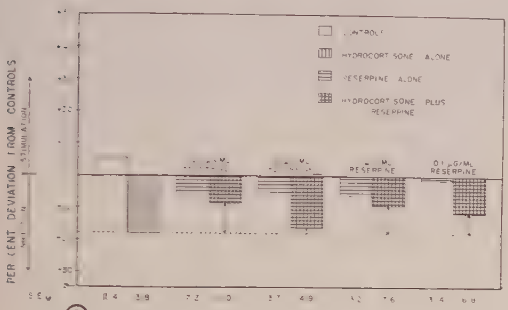

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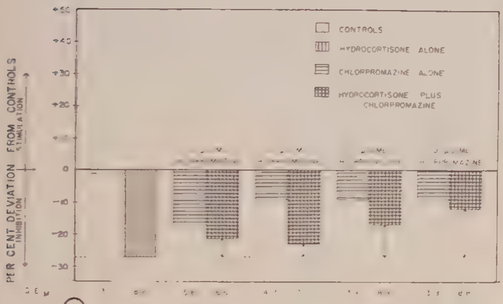

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Mixing vials 1 through 4 received both hydrocortisone and prochlorperazine. Mixing vials 5 through 8 received prochlorperazine alone. Mixing vial 9 received hydrocortisone alone; mixing vials 10 and 11 received no hydrocortisone and no prochlorperazine.

FIG. 1. Effects of prochlorperazine and hydrocortisone alone and in combination on leucocyte migration. Dotted line represents inhibition caused by addition of hydrocortisone alone, and arrows represent decrease in inhibition resulting from addition of specific concentrations of prochlorperazine to tubes containing hydrocortisone.

FIG. 2. Effects of reserpine and hydrocortisone alone and in combination on leucocyte migration. Dotted line represents inhibition caused by addition of hydrocortisone alone, and arrows represent decrease in inhibition resulting from addition of specific concentrations of reserpine to tubes containing hydrocortisone.

of 1 μg/ml of prochlorperazine is especially noteworthy This concentration caused average deviation from controls of –14% ± 2.6. Hydrocortisone alone, at a concentration of 100 μg/ml, caused average deviation from controls of –20% ± 3.1. Combination of 1 μg/ml of prochlorperazine and 100 μg/ml of hydrocortisone caused an average deviation from controls of –3% ± 0.8. Thus inhibition which occurs with either prochlorperazine or hydrocortisone alone is greatly reduced when the 2 are used in combination. A t-test comparing deviations from controls of capillary tubes containing hydrocortisone alone with those containing hydrocortisone plus 1 μg/ml of prochlorperazine shows that the difference is significant at the 0.05 level. Addition of 0.1 μg/ml of prochlorperazine caused an average deviation from controls of –18% ± 4.2, and the combination of hydrocortisone plus 0.1 μg/ml of prochlorperazine reduced the deviation to –11% ± 6.5.

It thus appears that inhibitions caused by prochlorperazine and by hydrocortisone are not additive. Indeed, at all concentrations tested, inhibition caused by hydrocortisone and prochlorperazine together was less than that caused by either one alone, and at a concentration of 1 μg/ml, prochlorperazine was able almost completely to reverse inhibition caused by hydrocortisone.

A further experiment was carried out to determine if a reaction was occurring directly between hydrocortisone and prochlorperazine, or whether each compound modified the reactions of the cell to the other. Earlier experiments (Ketchel and Sturgis; unpublished observation) showed that 1000 μg/ml of hydrocortisone was able to completely inhibit leucocyte migration. It was reasoned that if the reaction between hydrocortisone and prochlorperazine was a stachiometric one in which the leucocytes were not involved, then the effects of very large amounts of hydrocortisone could be reversed by addition of comparatively large

FIG. 3. Effects of thorazine and hydrocortisone alone and in combination on leucocyte migration. Dotted line represents inhibition caused by addition of hydrocortisone alone, and arrows represent decrease in inhibition resulting from addition of specific concentrations of thorazine to tubes containing hydrocortisone.

amounts of prochlorperazine. A series of cell suspensions containing 0.1, 1, 10, and 100 μg/ml was prepared, and hydrocortisone to attain a final concentration of 1000 μg/ml was added to each. In no case were lucocytes able to migrate in capillary tubes prepared from these cell suspensions, suggesting that reversal of hydrocortisone inhibition which occurred in experiments of Fig. 1 was not caused by a stachiometric reaction between hydrocortisone and prochlorperazine.

A similar series of 15 experiments was carried out with reserpine§ substituted for prochlorperazine (Fig. 2). Reserpine alone, even in concentrations as high as 100 μg/ml, was only slightly inhibitory to leucocyte migration. While at each concentration tested, average inhibition of reserpine plus hydrocortisone was less than that obtained with hydrocortisone alone, the differences did not have statistical significance. It is interesting to note that although statistical significance was not demonstrated, the average inhibition was less at all concentrations, when reserpine was included with hydrocortisone than with hydrocortisone alone. It seems clear that the inhibitions caused by hydrocortisone and by reserpine are not additive.

A third series of experiments was carried out using chlorpromazine hydrochloride‖ (Fig. 3). Thorazine alone was effective in inhibiting leucocyte migration in concentrations as low as 0.0001 μg/ml. As with reserpine, inhibition obtained by combining thorazine with hydrocortisone was less than that obtained with hydrocortisone alone although these differences were not large enough to be statistically significant. As with compazine and reserpine, inhibitions caused by thorazine and hydrocortisone are not additive.

Discussion. The present data indicate that prochlorperazine, at optimal concentrations, is able to reverse the inhibitory affect on leucocyte migration caused by hydrocortisone. Also, at other concentrations of prochlorperazine, and at all concentrations of reserpine and thorazine tested, the inhibitory effects are not added to those caused by hydrocortisone.

The importance of adrenal steroid hormones in the reaction of organisms to stress, is now well accepted, although the biochemical pathways through which the physiological responses are produced are poorly understood. Whatever the cellular mode of action of hydrocortisone, the present experiments suggest that it is interfered with by prochlorperazine, and perhaps by other tranquilizing agents.

Conclusions. 1. Addition to blood of 100 μg/ml of hydrocortisone or of 0.1 to 10 g/ml of prochlorperazine results in inhibition of leucocyte migration. Addition of both compounds together results in less inhibition than is obtained with either compound alone. 2. Reserpine at concentrations of 0.1 μg/ml to 100 μg/ml is less inhibiting to leucocyte migration than is prochlorperazine. Addition of both reserpine and hydrocortisone to blood results in less inhibition to leucocyte migration than when hydrocortisone alone is added. 3. Thorazine at concentrations of 0.001 μg/ml to 1 μg/ml is inhibitory to leucocyte migration. Addition of both thorazine and hydrocortisone to blood, results in less inhibition to leucocyte migration than when hydrocortisone alone is added.

1. Ketchel, M. M., Favour, C. B., Sturgis, S. H., *J. Exp. Med.*, 1958, v107, 211.

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3. Hanks, J. H., Wallace, R. E., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 196.

Received November 21, 1958. P.S.E.B.M., 1959, v100.

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§ Serpasil, Ciba Pharmaceutical Products, Summit, N. J.

‖ Thorazine, Smith, Kline and French, Philadelphia, Pa.

Effect of Foreign Fetal and Newborn Blood-Forming Tissues on Survival of Lethally Irradiated Mice. (24640)

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The "foreign bone marrow reaction" is a disease syndrome that occurs in lethally irradiated mice given homologous or heterologous (rat) bone marrow(1-5). The disease frequently, but not invariably, results in death from "metabolic starvation." Mice usually died 3 to 8 weeks after irradiation and bone marrow injection. A somewhat similar syndrome occurred in irradiated mice given infant homologous spleen cells instead of bone marrow(6). In certain genetic situations, delayed death pattern for lethally irradiated F_1 mice given bone marrow from one of the parent strains resembles that for mice dying from foreign bone marrow reaction(7-9). Delayed deaths, however, do not occur in all parent-F_1 combinations(10). Prevention or amelioration of foreign bone marrow reaction was first attempted by Cole *et al.*(2), who gave cortisone to lethally irradiated mice injected with rat bone marrow. These authors suggested that immunologic difficulties reduced effectiveness of rat bone marrow for treating irradiated mice. Cortisone did not substantially alter long-term survival. Evidence favoring immunologic basis for foreign bone marrow reaction was reviewed by Makinodan(11). Preliminary experiments by Congdon and Urso (3) indicated that homologous fetal blood-forming tissues were superior to homologous adult bone marrow for producing long-term survival of lethally irradiated mice. This finding has been confirmed in studies from this (12-14) and other laboratories. Lengerová (15) and Barnes *et al.*(16) also reported increase in number of long-term survivors among homologous radiation chimeras given fetal and newborn blood-forming tissues. Uphoff(17) found that long-term survival was more prevalent in irradiated F_1 mice given fetal, parental blood-forming tissues but few mice had long-term survival when given adult parental bone marrow. She also mentions a beneficial effect with homologous (C3H → AKR) fetal tissue not found with adult bone marrow (strain of mouse to left of arrow is the donor animal and to the right, the irradiated recipient). This mss. contains further evidence for superiority of homologous fetal and newborn bood-forming tissues as donor material in treatment of lethal whole-body radiation injury in mice of certain genetic types. Criteria for determining superiority include relatively long-term survival, histologic recovery of lymphatic tissues, and evidence that erythropoietic cells transplant and persist. Heterologous (rat) fetal blood-forming cells and adult rat bone marrow were about equally effective in producing long-term survival in lethally irradiated mice.

Materials and methods. Methods of collecting fetal blood-forming tissues from pregnant mice (12 to 19 days), and rats (12 to 21 days) and blood-forming tissues from newborn donors were similar. Suspensions of pooled liver or spleen tissue in Tyrode's solution were prepared by gentle aspiration through a syringe. After filtration through wire mesh screens, the suspension was passed through a 27-gauge needle. A sample of pooled tissue suspension was removed with red blood cell pipette and diluted with Wright's stain in 0.1 N HCl. Nucleated cells were counted, no differentiation being made between liver cell nuclei and blood-forming cells. A fetal mouse liver yielded 30 to 60 x 10^6 nucleated cells. The suspension was injected intravenously in the tail vein of recipient 1 to 6 hours after irradiation. To allow transfusion of large cell doses, 2 to 4 injections were given within a few minutes. Mice were observed several months, and number of deaths were recorded. Separate homologous and isologous transplantation experiments were performed for serial gross autopsy and histologic study of lymphatic tissue changes in lethally irradiated mice treated with adult bone marrow or fetal liver. Bone marrow, spleen, thymus, and lymph nodes were fixed in Zenker-formol, sectioned, and stained with hematoxylin and eosin. Recipient X-irradiated mice were 12-week-old males and females. Factors were 250 kv, 15 ma, 1 mm of Al added filtration, and hvl of 0.5 mm of Cu. Mice were given total-body irradiation of 900 or 950 r, 160 r/min at 60 cm, in revolving lucite cage. Homologous erythrocytes were identified by agglutination technic and a variation of Hildemann's hemolytic system(18).

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† Operated by Union Carbide Corp. for U. S. Atomic Energy Commission.

Results. Mortality after lethal irradiation of (C57L x A)F_1 mice given (101 x C3H)F_1 fetal liver is shown in Fig. 1. Control groups were given (101 x C3H)F_1 adult bone marrow or (C57L x A)F_2 fetal liver. It was not possible to obtain (C57L x A)F_1 fetal liver as true isologous control. Homologous fetal liver was more effective than adult homologous bone marrow in promoting 30-day and longer survival.

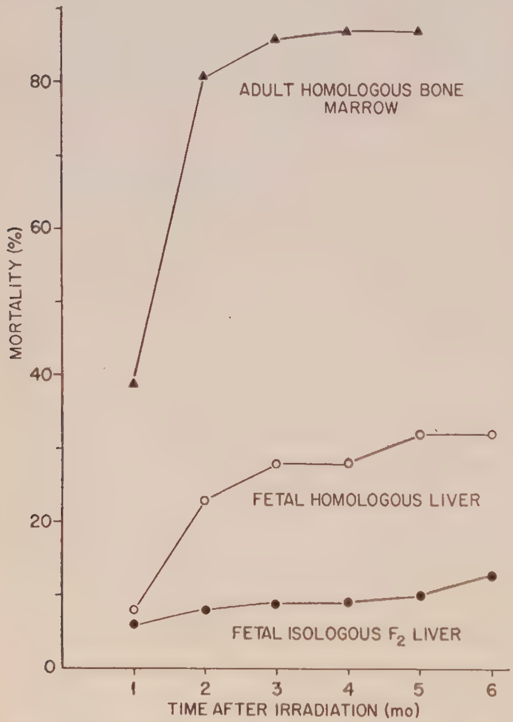

FIG. 1. Mortality in (C57L × A)F_1 mice given 900 r plus adult bone marrow or fetal liver. ▲, control group, 88 mice: dose, 30 to 50 × 10^6 (101 × C3H)F_1 adult bone marrow cells. ○, 47 mice: dose, 50 to 65 × 10^6 (101 × C3H)F_1 fetal liver cells. ●, 78 mice: dose, 38 to 60 × 10^6 (C57L × A)F_2 fetal liver cells.

In general appearance, behavior, rate of graying, mice given homologous fetal liver had a closer resemblance to those of isologous control group than to mice treated with adult homologous bone marrow.

Donor-type cells completely replaced red blood cells in 33 mice surviving homologous fetal liver treatment; about half the mice were tested 68 to 97 days after exposure, and the remainder at 123 to 130 days. Similar tests on 12 mice living 118 to 134 days after adult homologous bone marrow treatment, showed complete replacement by donor type cells.

Gross autopsy and histologic examination were performed on 20 (C57L x A) F_1 females given homologous (101 x C3H)F_1 fetal liver (80 x 10^6 cells) after 900 r. One mouse/day was killed and examined for first 7 days after treatment. This procedure was repeated at frequent intervals thereafter through day 63. One control group of 15 (C57L x A)F_1 females was given homologous (101 x C3H)F_1 adult bone marrow (30 x 10^6 cells) after 900 r. The same procedure was followed as in the experimental group, until day 32. The 17 (C57L x A)F_1 females in the other control group were given an equal amount of isologous adult bone marrow. Sacrifice and examination followed approximately the same plan as before from day 1 through day 57 after irradiation.

Mice given homologous fetal liver and those given adult homologous bone marrow, showed secondary loss in weight about 3 weeks after irradiation. At 4 weeks, body weight of all fetal tissue-treated animals had increased to that of mice given isologous bone marrow. 9 to 10 days after treatment, spleen weight in mice given each type of homologous cell treatment, paralleled that in mice treated with isologous bone marrow. Return to normal weight from extreme hyperplasia at day 10, did not occur after homologous cell treatment. With isologous bone marrow therapy, spleen weight returned to near normal levels within 3 weeks after exposure.

Histologic examination of mice given adult homologous bone marrow showed same changes in bone marrow, thymus, spleen, and lymph nodes previously reported for foreign bone marrow reaction(3). Granulomatous lymph node reactions leading to extreme atrophy were present within 7 days. Fibrinoid necrosis in white pulp of spleen was first seen in mice killed on day 10. Regional loss of bone marrow in the femur at day 32 indicated partial loss of graft at this site.

In contrast, mice given homologous fetal liver showed relatively mild granulomatous reactions in some lymph nodes. These reactions regressed or persisted to a very limited extent. Extreme atrophy of nodes was not observed, and normal regeneration was striking. At 32 days, lymph node structure was normal. Splenic white pulp also returned to normal appearance. Fibrinoid necrosis was not seen. After 50 days, lymph nodes and splenic white pulp did not appreciably differ from these tissues in irradiated controls given isologous bone marrow.

Further to check these morphologic findings, we studied 2 other genetic combinations. In each instance, mice treated with homologous fetal liver were compared with those given homologous adult bone marrow. In one experiment, each of 22 irradiated (900 r) (C57L x A)F_1 mice was given 103 x 10^6 fetal liver cells from BDF_1 mice, and 22 were each given 40 x 10^6 bone marrow cells from adult BDF_1 donors. At frequent intervals from 1 to 64 days after exposure, some mice were killed and autopsied. In the other experiment, 16 irradiated (950 r) (101 x C3H)F_1 mice were each given 70 x 10^6 fetal liver cells from BDF_1 mice, and 18 were each given 39 x 10^6 bone marrow cells from adult BDF_1 mice. Mice were killed and autopsied at frequent intervals from 1 to 50 days in the fetal group and 1 to 64 days in the adult.

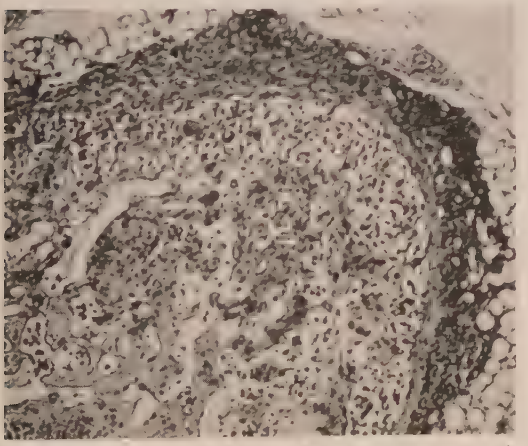

FIG. 2. Atrophic skeleton of a peripheral lymph node from a (C57L × A)F_1 mouse 50 days after 900 r and BDF_1 adult bone marrow treatment. Hematoxylin and eosin × 40.

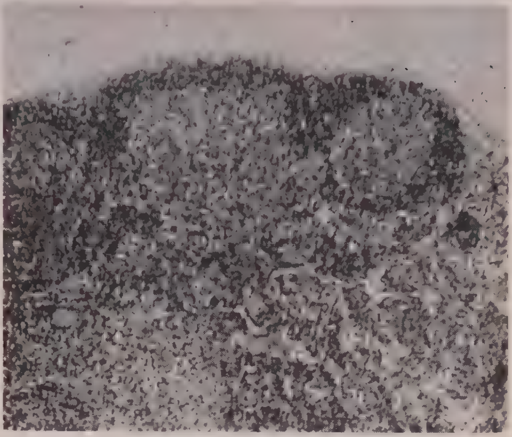

FIG. 3. Normal appearing peripheral lymph node from a (C57L × A)F_1 mouse 50 days after 900 r and BDF_1 fetal liver treatment. Hematoxylin and eosin × 40.

In both genetic combinations, autopsy and histologic studies indicated great superiority of homologous fetal liver treatment over that of adult homologous bone marrow. Figs. 2 and 3 show lymph node recovery in 1 experiment. The so-called 3-day swelling reaction of splenic white pulp was much weaker with homologous fetal liver(5) than with adult homologous bone marrow.

In another experiment of same design, equal amounts (22 x 10^6 cells) of isologous fetal liver or adult bone marrow were given to irradiated (950 r) (101 x C3H)F_1 mice. No significant differences between adult and fetal isologous tissue therapy were observed at autopsy and histologic study.

In other survival studies, pooled (101 x C3H)F_2 fetal liver was greatly superior to pooled (101 x C3H)F_2 adult bone marrow for treatment of lethally irradiated (C57L x A)F_1 mice. Similarly, survival was higher in irradiated (C57L x A)F_1 mice given BDF_1 fetal liver than in those given BDF_1 adult homologous bone marrow. There were fewer delayed deaths, however, in this strain with BDF_1 adult homologous bone marrow than with (101 x C3H) F_1 or F_2 adult homologous marrow.

Fetal homologous spleen and liver were about equally effective in preventing delayed deaths in 11 (C57L x A)F_1 mice. One-day-old newborn homologous spleen (36 mice) or liver (32 mice) was as effective as fetal tissue in preventing delayed deaths. However, 2- to 4-day-old newborn homologous liver (33 mice) and 2- to 6-day-old newborn homologous spleen (53 mice) caused many delayed deaths, comparable in number to those resulting from treatment with adult homologous bone marrow. All homologous donors in these experiments were (101 x C3H)F_1 or F_2 mice. Isologous fetal and newborn liver and spleen in (101 x C3H)F_1 mice, promoted survival from radiation injury, as found earlier by Jacobson *et al.*(19) and Duplan(20).

In preliminary experiments, studies with fetal homologous liver cells gave no obvious significant differences in survival for 5 months after irradiation. Cell doses ranged from 19 to 117 x 10^6 nucleated cells. Donors at all stages of gestation were included. Donor livers were from (101 x C3H)F_2 mice, and 152 recipients were (C57L x A)F_1 mice. The long-term survival of mice treated with homologous fetal liver cells (30 to 60 x 10^6), was not significantly affected by gestation age of donor; cells taken at 12 to 17 and 16 to 20 days of gestation had about the same effect. The 72 recipient mice were the same types as those used for cell-dose study.

Fetal and newborn blood-forming tissues from Sprague-Dawley rats were given to male and female (101 x C3H)F_1 mice after 950 r for comparison with the effect of adult rat bone marrow treatment. Cell doses of fetal (15 to 22 days of gestation) and 1-day-old newborn rat liver (equal to dose of adult rat bone marrow), did not increase the number of 30-day (and longer) survivors (Fig. 4). Rather low 30-day survival was obtained with somewhat smaller doses of fetal and newborn (1- to 6-day-old) rat spleen. Rat red blood cell chimerism was observed in 7 mice tested 240 days after rat fetal liver treatment by hemagglutination technic described by Makinodan(21).

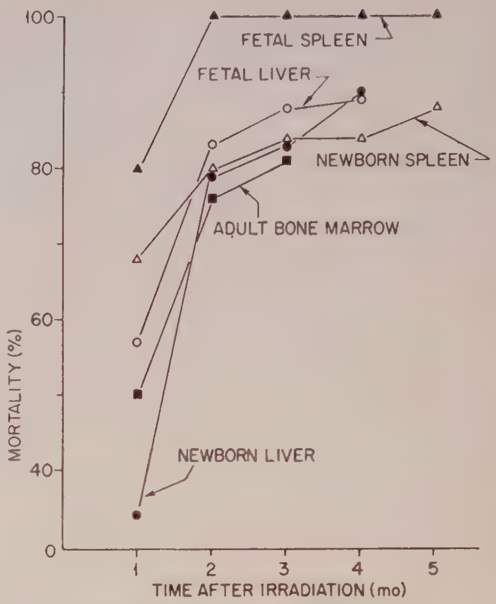

FIG. 4. Mortality in (101 × C3H)F_1 mice given 950 r plus rat fetal or newborn liver or spleen after irradiation. ■, controls, 101 mice: dose, 50 to 180 × 10^6 adult bone marrow cells. ○, 161 mice: dose, 80 to 180 × 10^6 fetal liver cells. ●, 29 mice: dose, 60 to 120 × 10^6 newborn 1-day-old liver cells. ▲, 10 mice: dose, 8 to 50 × 10^6 fetal spleen cells. △, 25 mice: dose, 10 to 60 × 10^6 newborn (1- to 6-day-old) spleen cells.

Discussion. Our observations show that therapy with fetal and 1-day-old newborn homologous liver and spleen, alters foreign bone marrow reaction in mice of these genetic combinations. Reports by Uphoff(17), Lengerová(15), and Barnes *et al.*(16) indicate that fetal liver and spleen in other genetic combinations are also capable of altering delayed mortality in foreign bone marrow experiments.

Owen and Urso did not find good long-term survival in C57BL/10D2 → (C3H x 101)F_1 mice treated with homologous fetal liver. (D. Uphoff and J. Trentin have also commented on genetic homologous combinations in which fetal blood-forming tissues were not superior to adult bone marrow in promoting long-term survival after irradiation.) Fetal rat liver was not superior to adult rat bone marrow cells when used in irradiated mice. More extensive investigation into cell-dose requirement, age of gestation, and specific histocompatibility relations are probably needed for understanding of these unfavorable results.

Porter and Moseley(22) used 1- to 3-day-old newborn rabbit liver in irradiated homologous rabbits with 50% 14-day and 25% 56-day survival. This therapy seems inferior to previous results reported by Porter and Murray(23) with homologous rabbit bone marrow. Lacassagne *et al.*(24) had few survivors among irradiated rats, given a small dose of rat fetal liver.

Primary mechanism of action in favorable experiments with fetal and 1-day-old newborn tissues, is of considerable importance. Reports by Uphoff(17), Lengerová(15) and Barnes *et al.*(16) suggest a reverse "actively acquired tolerance" in which grafted fetal cells are presumed to have no antibody-forming ability. When antibody-forming cells do develop from the graft, they become "tolerant" of host antigens. The mechanism of actively acquired tolerance is itself not understood, and hence this becomes a kind of experimental analogy. Antibody-forming potentiality of blood-forming cells in fetal liver is not known.

Another idea for a primary mechanism would be the possibility of antigenic immaturity of fetal cells. These would be less able to evoke an immune reaction in the irradiated host.

In these experiments, fetal liver cells had no obvious effect on liver or other tissues of irradiated host.

Summary. Lethally irradiated mice that received blood-forming cells from homologous fetal liver showed better long-term survival and lymphatic tissue recovery, than those treated with blood-forming cells from adult homologous bone marrow. In both cases there was transplantation and persistence of erythropoietic cells.

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Growth Hormone and Thyroxine: Effects on Submaxillary Gland of Hypophysectomized Rat.* (24641)

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Considerable work indicates that thyroid function and submaxillary gland histology and physiology in the rat are intimately related, and that testosterone is synergistic with thyroid hormone in maintaining normal histologic appearance and enzymatic activity of submaxillary glands(1-5). Shafer *et al.*(4) indicated that either cortisone or growth hormone may also increase proteolytic activity of salivary glands of hypophysectomized rats but to a lesser degree than thyroxine. Subsequently, cortisone revealed that it actually inhibited the effects of thyroxine, in maintaining normal salivary function and enzymatic activity. The present study is concerned with a more detailed investigation of the effects of growth hormone, both alone and in combination with thyroxine, upon rat submaxillary gland.

Materials and methods. Forty male albino rats, hypophysectomized‡ at 4 months of age, were equally divided into 4 groups. Group I received daily subcutaneous injections of growth hormone§ (1 mg/kg reconstituted in distilled water). Group II was given 20 mg of sodium thyroxine subcutaneously each day, and Group III growth hormone and thyroxine in same concentrations as used above. Group IV received no hormones or special supplements. Six unoperated rats of same age were controls. All 46 animals received *ad lib.* laboratory chow supplemented by special diet of bread, oranges, carrots and milk. After 24 days animals were sacrificed by chloroform and one submaxillary gland from each animal was assayed for proteolytic activity(4). The remaining submaxillary gland was fixed in Bouin's solution for histologic study. Parotid gland, stomach, pancreas, adrenal and thyroid glands were also fixed for histologic study. Paraffin sections of glands were cut at 8 μ and stained by (1) hematoxylin and eosin; (2) methyl green-pyronine for ribonucleic acid(7); and (3) periodic acid-Schiff method. Additional sections from each gland were incubated in sterile solution of crystalline ribonuclease‖ (0.25 mg/ml for 2 hours at 37°C), then stained with methyl green-pyronine.

Results. Table I shows the effect of various hormone supplements upon body weight. These data confirm our original observation that growth hormone is effective in maintaining normal body weight in the hypophysectomized rat. Thyroxine administration, on the other hand, resulted in an even greater weight loss than that observed in non-treated hypophysectomized rat. The effect on body weight of thyroxine and growth hormone administered together indicates that weight-gain inhibitory effects of thyroxine predominate since these animals gain weight at only a minimal rate in spite of growth hormone supplements.

Measurements of the diameter of submaxillary gland tubules indicate that hypophysectomy markedly reduces size of tubules. Administration of either thyroxine or growth hormone only partially prevents atrophic changes (Table I). The 2 hormones administered together maintained normal tubule size. Similarly, atrophic histologic changes seen throughout the submaxillary gland of the hypophysectomized rat were only partially prevented by either thyroxine or growth hormone alone, while the 2 in combination produced histologic appearance indistinguishable from the normal. Such results are unexpectedly similar to those previously published concerning the efficacy of thyroxine and testosterone in preventing hypophysectomy-induced submaxillary gland changes(4,6).

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† Post Doctoral Fellow, Nat. Inst. of Health, Division of Dentistry.

‡ Hormone Assay Lab., Chicago, Ill.

§ Growth hormone was generously supplied by Dr. Irby Bunding, Armour Labs., Chicago, Lot #M208.

‖ Previously heat-treated at 90°C for 10 min. to destroy any contaminating desoxyribonuclease.

TABLE I. Effect of Growth Hormone and Thyroxine upon Body Weight, Proteolytic Activity, and Submaxillary Gland Tubules in the Hypophysectomized rat.

	No. animals surviving	Final body wt (g)	Wt change (g)	Diam. of submax. tubules	Avg wt submax. gland (mg)	Submax. wt (mg/100 g body wt)	Relative activity†	Activity/g fresh tissue‡
Control	5	345	+40	44.3 ± 1.7*	270.3	77.8 ± 4.7*	12.051	9.086 ± .678*
Hypox	9	240	−27	30.7 ± .7	143.7	59.8 ± 4.4	2.308	3.282 ± .296
Hypox + GH	8	305	+41	38.1 ± 2.8	194.2	63.7 ± 3.8	4.734	4.951 ±1.254
Hypox + thyroxine	8	220	−32	40.1 ± 2.2	165.2	76.5 ± 4.5	4.181	5.163 ± .958
Hypox + GH + thyroxine	7	284	+17	45.3 ± 1.2	251.4	88.6 ± 4.0	11.085	8.988 ±1.472

* Stand. dev.

† Expressed as ratio between color produced by total gland and color of tyrosine standard.

‡ Expressed as ratio between color produced/g of gland tissue and activity of tyrosine standard.

The effect of hormone supplements on submaxillary gland weight is also shown in Table I. Hypophysectomy markedly reduced gland weight, and growth hormone was ineffective in preventing weight loss. Thyroxine, in contrast, was completely effective in preventing submaxillary gland weight loss. Apparently growth hormone is effective in maintaining body weight but has little effect upon submaxillary gland weight. Thyroxine, on the other hand, maintains submaxillary gland weight while it is ineffectual in maintaining body weight.

Table II also indicates alterations observed in proteolytic activity of the submaxillary gland following hypophysectomy and hormone treatments. Hypophysectomy reduced proteolytic activity by about 65% as compared to control values. Administration of either growth hormone or thyroxine alone, was partially effective in maintaining normal proteolytic activity. Only combined administration of growth hormone and thyroxine resulted in submaxillary gland proteolytic activity within the normal range. Shafer *et al.* (4) reported that of many hormones tested only testosterone and thyroxine together produced normal submaxillary proteolytic activity values in the hypophysectomized rat. However, combinations of thyroxine with other hormones were not attempted. In this study, combination of growth hormone and thyroxine seems quite comparable to that of testosterone and thyroxine. The reason for this result remains obscure. Perhaps the preparation of growth hormone used contained a trace of gonadotrophic hormone which had a stimulatory action on gonadal tissue. However, it seems equally probable that the effect is merely a metabolic one in that growth hormone and testosterone may help to restore a general metabolic balance to the hypophysectomized animal.

It is important to know what effects growth hormone and thyroxine have upon other organs. Ribonucleic acid content of the sub-

TABLE II. Location and Distribution of Ribonucleic Acid in Organs of Hypophysectomized Rat Treated with Growth Hormone and Thyroxine.

	Submaxillary gland*		Pancreas†		Stomach‡	
	Conc.	Location	Conc.	Location	Conc.	Location
Control	+++	BP	+++	PB	++	P
Hypox	+	B	+	P	+	P
GH	+	B	+(+)	P	+	P
Thyroxine	++	BD	++	PD	+	P
GH + thyroxine	+++(+)	BPD	+++	PD	++	PD

* Found only in acinar cells, no RNA evident in duct cells except nucleolus.

† " " " " " , none in islet cells except nucleolus.

‡ " " " zymogenic cells. Parietal cells exhibited little if any RNA except in nucleolus.

B—Basal, D—Diffuse, P—Perinuclear.

Enzymes of *Entamoeba hisolytica* I. Gelatinase.* (24642)

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Relatively little is known regarding the enzymatic makeup of *Entamoeba histolytica*. Amylase(1-3), phosphomonoesterase(4), glutaminase(5), maltase(2), esterase(6), succinic dehydrogenase(7), and proteolytic enzymes (8-10) have been reported in cultural forms of the amoeba. Since it is necessary to determine enzyme constitution of an organism to elucidate its metabolic pattern, it was considered important to make a systematic study of enzymes in *E. histolytica*. It is realized that enzymes present in amoebae *in vivo* may not be produced in culture. A case in point would be hyaluronidase, which is absent in amoebae maintained in cultures over a long time, yet has been detected in hamster liver abscesses (11). Until an axenic culture of *E. histolytica* becomes available, many studies dealing with enzymes, metabolism, and growth requirements will be beclouded by the activity of associate bacteria, minimal though it may be. However, with adequately controlled experiments, vital information can be obtained which may directly or indirectly lead to cultivation of amoebae in pure culture freed of all other living systems. In the study of enzymes, fairly large amounts of cell material are required; the bacteria-inhibited, growth-factor fortified culture method recently developed by Nakamura(12) seemed appropriate for obtaining large numbers of amoebae, which can be further purified by repeated washing and centrifugation. The reevaluation of enzyme gelatinase appeared valuable since information regarding the role of protein in amoebic nutrition is so poorly understood and also because information on proteolytic enzymes of *E. histolytica* may lead to a new approach regarding the pathogenic component of this protozoan parasite(13). Since most earlier work on presence of gelatin-hydrolyzing enzymes was performed with tubes containing high concentration of gelatin, some doubt exists as to whether liquefaction of gelatin was due to amoebic or bacterial associate activity. Employing a sensitive microtest for gelatinase developed by Thirst(14) and an agar-gelatin plate test developed by Smith(15), 6 strains of *E. histolytica* were studied for their ability to hydrolyze gelatin. Numerous control experiments and variations of the tests were included to show unequivocally that enzyme activity was due to amoebae.

Materials and methods. Six strains of *E. histolytica* (HUS-105, HUS-100, Washington, Griffith, ROK, and Conrad) were used. The HUS-100 and HUS-105 strains, both large races, were obtained from Dr. Chia-tung Pan; the other strains, all small races, were obtained from Dr. Russell M. McQuay, Jr. The amoebae were routinely carried on coagulated egg slants overlaid with Ringer-serum solution. Several methods were employed for detection of gelatinase. The microtest consisted of a thin film of gelatin as substrate on a glass slide; slides were prepared by dipping clean, dry microscope slides into melted aqueous gelatin solutions containing phenol, draining, and drying. Suspensions of amoebae were placed on the slides, which were incubated at 20°C for 12-24 hours in Petri dishes containing strips of moistened filter paper, to minimize evaporation. Hydrolysis of gelatin on these slides was indicated by appearance of clear areas on the slide, when stained with dilute carbolfuchsin, a protein stain. The presence of phenol prevented bacterial contamination without affecting gelatinase activity. The second method consisted of preparing agar places containing 0.4% gelatin upon which was placed the material to be assayed. Large drops of amoebae in suspension were placed on the surface of the gelatin-agar plates. The

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plates were incubated at 37°C for 12-24 hours, then flooded with approximately 10 ml of a solution of mercuric chloride in hydrochloric acid, a gelatin precipitant. A white precipitate indicated presence of gelatin and absence of precipitate (clear areas on plates) indicated gelatin hydrolysis. Amoeba materials assayed were prepared in several ways: (a) amoebae grown in absence of multiplying bacteria, with the aid of growth factors, adenosinetriphosphate and ribose-5-phosphate, and large concentrations of antibiotics, were pooled and washed with Ringer solution 5-7 times and finally made up into standard suspension containing 200,000 to 250,000 amoebae/ml; (b) pooled amoebae from stock cultures grown in association with a mixed bacterial flora were washed 6 times; portions of supernate from third and sixth washings and the sediment from sixth washing were assayed; (c) *E. histolytica* grown with *Escherichia coli* (a monobacterial associate) was used; *E. coli* does not hydrolyze gelatin. Control experiments consisted of: (a) placing drops of Ringer solution on slides and plates; (b) use of *Proteus vulgaris* and trypsin to detect gelatin hydrolysis; (c) inoculation of associated flora from amoeba cultures into conventional gelatin tubes to detect liquefaction of gelatin by the mixed bacterial flora; (d) destruction of amoebic gelatinase by autoclaving and inhibiting the enzyme with 20% copper sulfate and (e) alteration of enzymatic activity by alternate freezing and thawing of amoeba suspensions. Finally, to determine optimum pH for amoeba gelatinase by studying the enzyme at different pH values using phosphate buffer solutions.

Results. All 6 strains of *E. histolytica* hydrolyzed gelatin. The mixed bacterial flora were also capable of hydrolyzing gelatin. However, the aboebae were washed 5-7 times and the supernatant from final washing, which could conceivably contain a few bacterial cells, did not hydrolyze gelatin; therefore, it is evident that the activity was due to amoebae alone and not due to residual bacterial enzyme. After third washing, the supernate was without gelatinase activity. The amoebae also hydrolyzed gelatin when grown in a monoassociated culture with *Escherichia coli,* a species known not to hydrolyze gelatin. Amoebic gelatinase activity was inhibited by heat, copper sulfate, and freezing and thawing. The washed amoeba suspensions were diluted with saline to obtain a ½, ¼, ⅛, 1/16, and 1/32 dilution and tested for gelatinase activity on gelatin-agar plates. The degree of hydrolysis decreased with increased dilutions so that a 1/16 dilution showed only a faint zone of hydrolysis, and the 1/32 dilution showed no hydrolysis. Gelatinase activity of amoebae was not affected within a pH zone of 6.5 to 8; there was a decrease in activity at pH of 5. The pH values below 5 and above 8 could not be studied due to gelatin hydrolysis caused by acidity and alkalinity.

Summary. Six strains of *E. histolytica* were shown to hydrolyze gelatin. The gelatinase was inactivated by heat, copper salts, and freezing. The effects of concentration of enzyme and pH were studied.

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Reduction of X-Radiation Mortality by Cabbage and Broccoli.* (24643)

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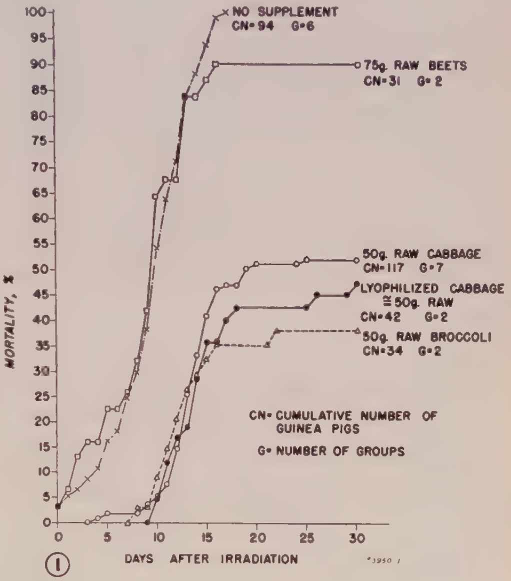
In studying composition of diet as one of the factors contributing to large variation in resistance of guinea pigs to whole body X-radiation, Lourau and Lartigue(1) reported significantly higher mortality and incidence of hemorrhages in animals receiving supplement of beets, than in those receiving supplement of cabbage. (An unsupplemented control group was not available.) They considered 2 possible explanations: cabbage is protective and diminishes incidence of hemorrhages because of high content of Vit. P and C; or, beets contain a substance perfectly tolerated by normal animal, but which becomes toxic to an irradiated animal. They adopted the latter explanation. This observation led us to perform experiments to test these reported effects of cabbage and beets and to investigate ability of other foods to increase or decrease mortality due to radiation exposure.

Materials and methods. Young male albino guinea pigs, weighing 250 to 325 g, were maintained on basal diet of equal parts of whole field oats and wheat bran, *ad lib.* Supplements of beets (75 g), cabbage (50 g) or broccoli (50 g) were given to at least 2 groups of animals. Raw vegetables were diced and given in separate cups.† Lyophilized cabbage was wetted with twice its weight of distilled water. After 2 weeks of pre-feeding, all animals were exposed to 400 r of whole-body X-radiation. Irradiation was carried out at Argonne National Laboratory‡ using a General Electric Maximar X-ray machine. Radiation factors were 180 KV, 15 ma, no filtration



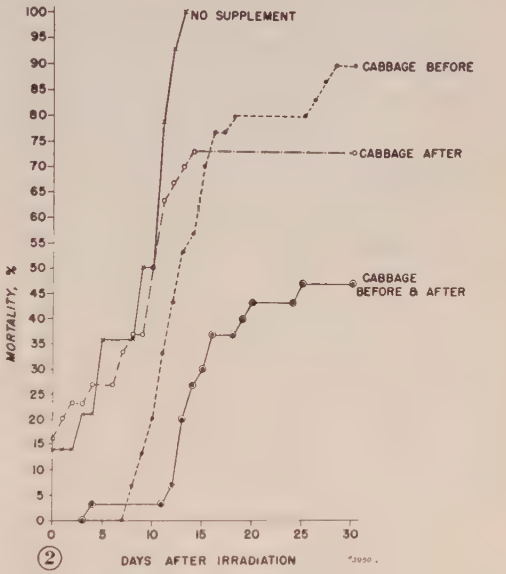


FIG. 1. Effect of supplementation with beets, cabbage, and broccoli on mortality of 224 guinea pigs exposed to 400 r of whole-body X-radiation.

FIG. 2. Influence of time of cabbage supplementation, before or after irradiation, on mortality of 90 guinea pigs exposed to 400 r of whole-body X-radiation.

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* This paper has been assigned No. 907 approved for publication. Views or conclusions are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the Dept. of Defense.

† Beet tops, cabbage core and tough portion of broccoli stalk were discarded.

‡ We wish to thank Dr. D. E. Smith, Joseph Trier and Emil Johnson of Argonne National Lab. for irradiation of guinea pigs.

TABLE I. Body Weight before and after Irradiation in a Typical Experiment.

Supplement	No. of guinea pigs	Avg body wt (g) on days before and after irradiation −4	0	+3	+7	+10	+14	+21	+31
					Survivors				
None	0								
Cabbage, 50 g	8	304	319	310	310	309	290	286	321
Broccoli, "	13	310	334	330	340	339	339	366	400
					Decedents				
None	20	276	279	258	260(19)*	264(14)	236(10)		
Cabbage, 50 g	12	301	320	303	311	292	277 (7)		
Broccoli, "	7	305	323	311	305	288 (6)	216 (2)		

* Numbers in parentheses are for this time period, as death intervened.

added, 21-22 r/minute, 41 inches target distance. Six animals were irradiated simultaneously in horizontal beam and each exposure included representatives from each group. Half of the dose was applied to each side of animals.

Results. One hundred percent of 94 guinea pigs fed only the basal diet died within 10 to 15 days after irradiation (Fig. 1). Supplementation with beets did not affect mortality rate of irradiated guinea pigs during the 30-day test. Feeding raw cabbage significantly reduced mortality in all 7 trials to average of 52% (range, 20 to 64%)[§] Lyophilized cabbage was fed at level equivalent to 50 g of raw cabbage, and retained ability to reduce radiation mortality. Another member of the *Brassicaceae* family, *i.e.,* broccoli, was found in 2 trials to be even more effective than cabbage in reducing radiation mortality. Analysis of variance of proportions of animals killed during the 30-day test (using arc sine transformation) showed that the difference in mortality due to feeding of cabbage or broccoli, was highly significnt ($p < .001$) (2).

We then determined separate effects of pre- and post-irradiation feeding of cabbage. Significance of results was evaluated by analysis of variance of cumulative proportions of animals killed at each time period (using arc sine transformation) averaged over 30 days. Prefeeding of guinea pigs with cabbage just prior to radiation exposure, significantly ($p < .001$) delayed onset of death and enabled some animals to survive the test period (Fig. 2). Post-irradiation supplementation with cabbage yielded significantly ($p < .001$) lower total mortality. The greatest number of survivors resulted from feeding of cabbage both before and after radiation exposure.

Average consumption of basal bran and oats diet was 14 g/day by control groups and by guinea pigs fed a vegetable supplement. Irradiation had a relatively small effect on food consumption. Intake of basal diet was about 60% of normal for first 4 days and gradually increased to pre-irradiation level by 21st day. Intake of cabbage dropped 3 to 5 g between 8th and 20th days. Body weight changes followed a typical pattern of initial loss on day 1, followed by gradual recovery until 8th-post-irradiation day, when a second period of weight loss occurred, persisting until 13th day. Representative changes in body weight are shown in Table I. Some mortality occurred at time of initial weight loss, the major incidence of death, however, was simultaneous with second period of weight loss, between 11th and 13th days. Animals which survive this second phase, in general, survive the 30-day test.

Protective effect of cabbage supplementation has also been demonstrated in another species, the rat (unpublished), maintained on standard synthetic ration which is nutritionally adequate in all known nutrients. Studies are in progress to explore these important findings by survey of wide variety of foods, which increase or decrease sensitivity of rats and guinea pigs to radiation injury and to ascertain whether these effects occur in other species of animals.[‖]

[§] In exploratory trials ascorbic had no influence on mortality and was therefore included in drinking water of all animals at level of 75 mg/100 ml.

[‖] Acknowledgement is made to the following for assistance in these experiments: Dr. Leonard Sheffner, Lucius Thomas, Gladys Eckfeldt and Robert Potts.

Summary. Exposure to 400 r of whole-body X-radiation resulted in 100% mortality in 10 to 15 days of young male guinea pigs fed a basal diet of bran and oats plus ascorbic acid. Supplementation with cabbage or broccoli for 2 weeks before irradiation and during 30 days after irradiation significantly reduced mortalty. Lyophilized cabbage retained ability to reduce radiation mortality. Pre-feeding of guinea pigs to time of radiation exposure, signficantly delayed onset of death and enabled some animals to survive test period. Post-irradiation supplementation with cabbage also yielded a lower total mortality. Feeding of cabbage both before and after radiation exposure produced greatest amount of protection.

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Received December 2, 1958. P.S.E.B.M., 1959, v100.

Response of Ciliated Epithelia of Different Histological Origin to Virus Infections *in vitro*. (24644)

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We recently reported(1) that ciliated epithelia of the respiratory mucosa of man or monkey, exposed *in vitro* to polio- or adenoviruses, can maintain for relatively long periods their morphological and functional integrity. This phenomenon is especially evident in mixed cultures in which histologically organized epithelium is associated with dedifferentiated cells of the same species, such as HeLa or monkey kidney cells. These cells are selectively destroyed, leaving organized ciliated epithelium unharmed, in spite of high virus concentrations. The present experiments were performed to determine whether: (1) the observed phenomenon occurs also in ciliated epithelia of histological location other than respiratory tract, (2) this phenomenon can be demonstrated also with highly epitheliotropic viruses, such as herpes simplex virus, (3) the observed resistance to virus infections can be abolished by chemical or enzymatic treatment of ciliated cells.

Material and methods. Cultures of plasma imbedded rabbit or monkey trachea fragments were prepared and processed in living state or after fixation and staining, according to previously described methods(1). Cultures of rabbit Fallopian tube fragments were cut from upper convoluted oviduct, including its fimbriated extremity. Human ovarian tubes were obtained by total hysterectomy in 5 cases of uterine cancer on patients 40-56 years of age. No histological evidence of malignancy was present in the Fallopian tubes. Only fimbriated extremity of human oviducts was used for explantation. In certain experiments HeLa cells or 2nd and 3rd passage rabbit kidney cells were added to cultures of Fallopian tube fragments as previously described(1). Culture medium contained 0.5% lactalbumin hydrolysate in Earle's solution, supplemented with 0.1% yeast extract, 15% decomplemented horse serum and 3% bovine embryo extract. Poliovirus type 1 Mahoney strain, adenovirus type 1 "Ad 71" strain,[†] tissue culture adapted strain of Mengo encephalomyelitis virus(2), "5433" strain of herpes simplex virus(3), infectious bovine rhinotracheitis virus (I.B.R.)[‡] and a strain of vaccinia virus isolated from commercial calf lymph vaccine, were used. Virus titrations were performed by serial dilutions in tube cultures of trypsinized rabbit kidney cells for herpes simplex virus and HeLa cells for other viruses.

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[*] U. S. Government Fulbright fellow.

[†] Received from Dr. R. Huebner, N.I.H., Bethesda.

[‡] Received from Dr. Cabasso, Lederle Laboratories, Pearl River, N. Y.

TABLE I. Behavior of Human Fallopian Tube and HeLa Cell Cultures Infected with Different Viruses.

Tissue culture	Age of culture*	Virus	ID_{50} introduced per culture	Max time of survival after infection	Virus titer attained† ID_{50}
Org. C. Ep.	48 hr	Polio	10^8	7 days	$10^{4.5}$
" ‡	"		"	6	10^6–10^7
Gr. Ep.‡	"		"	3	"
HeLa	"		"	48 hr	10^7
Org. C. Ep.	4 days		10^6	>7 days	
"	Fresh		10^5	9	>10^5
"	3 days	Mengo	"	>8	
" ‡	8		"	"	10^6
Gr. Ep.	"		"	6	"
HeLa	3		"	48 hr	$10^{7.5}$
Org. C. Ep.‡	4	I.B.R.	10^4	>10 days	10^5
"	13		"	"	
HeLa	3		"	4	"
Org. C. Ep.	3	Adeno type 1	"	12	
Gr. Ep.	"		"	5	
HeLa	"		"	3	

Org. C. Ep. = Organized ciliated epithelium present on explanted fragments.
Gr. Ep. = Dedifferentiated epithelium of outgrowth area.

* At time of virus introduction.
† Per ml of supernatant at time indicated as maximum survival.
‡ Mixed Fallopian tube and HeLa cultures.

Results. General aspect of Fallopian tube cultures. In most explanted fragments of adult rabbit and human oviducts, surface sheets of ciliated epithelium maintained characteristic histological structure and coordinated, vibratory activity usually for 20-25 days. In some fragments it lasted 40 to 50 days after explantation. The outgrowth, composed chiefly of confluent sheets of epithelial cells, could be maintained in culture for several months and in some cases produced permanent cell lines.§ Isolated spots of ciliated cells were present frequently in the outgrowth, but their vibratory activity was less persistent than in fragments.

Human oviduct cultures infected with polio and other viruses. Table I shows the behavior of human oviduct cultures, and of HeLa cell cultures, infected with polio, Mengo encephalomyelitis and adenoviruses. As a general rule, organized ciliated epithelium reacted to virus infections, if at all, only after considerable delay as compared with dedifferentiated HeLa cells or cells present in outgrowth area. This difference was particularly conspicuous in mixed cultures where a perfectly active ciliated epithelium could be observed many days after complete destruction of all dedifferentiated cells.

Rabbit oviduct cultures infected with herpes simplex and other viruses. The "5433" strain of herpes simplex virus (105th-108th *in vitro* passages in rabbit kidney cell cultures) when introduced in concentrations of 10^4-10^5 ID_{50}/ml in dedifferentiated rabbit epithelial cell cultures, caused lesions characterized by generalized giant cell formation§ and specific nuclear changes resulting in complete destruction of cultures in <48 hours. Fragments of rabbit Fallopian tubes incubated 1 hour with 10^4 ID_{50}/ml virus suspension, then washed and explanted, showed for first 3 days apparently normal growth and cell migration. On 4th day specific cell degeneration started at periphery of epithelial outgrowth and became generalized on 5th day. However ciliary activity continued undisturbed until the 6th day. At this time in fixed preparations, specific herpes nuclear lesions could be seen in all cells of outgrowth area, in some cells of epithelial lining of the fragment, among them an occasional ciliated cell. In other experiments, rabbit Fallopian tube cultures grown in association with homologous dedifferentiated kidney cells, were infected with virus after 2, 5,

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§ Unpublished data.

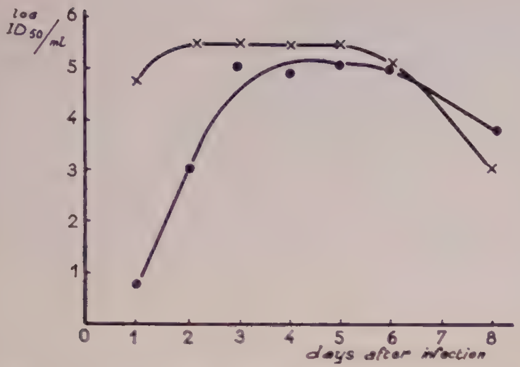

FIG. 1. Herpes virus concentration in supernatant of infected rabbit kidney dedifferentiated cell cultures (—×—) and cultures of rabbit Fallopian tube fragments (——●——). Non-adsorbed virus eliminated by washing at time 0.

7 or 10 days of preliminary culture. In these cultures outgrowth and associated cells were entirely destroyed in <3 days after infection. This was accompanied in every case by sustained virus titer in the medium of 10^5-10^6 ID$_{50}$/ml. In all these cultures ciliary activity could be observed for at least 48 hrs after complete destruction of the nonorganized growth. Later, the ciliated lining epithelium was progressively invaded and destroyed. Similar observations were made on rabbit trachea cultures. Comparison of curves of virus liberation in trypsin dispersed rabbit kidney cell cultures and cultures of rabbit Fallopian tube fragments (Fig. 1) shows that the virus was produced in both cases, but reached maximum levels much more rapidly in dedifferentiated kidney cells.

Rabbit oviduct cultures infected with vaccinia behaved in a similar way with herpes virus.

Virus action on chemically or enzymatically pretreated ciliated cells. It is possible that mechanical activity of cilia is by itself able to disturb fixation and penetration of virus into the cells. To check this possibility we tried, before bringing the cells in contact with the virus, to stop ciliary activity in a reversible way with appropriate anesthetics. Nembutal (Na methyl-butyl-barbiturate) acting in concentrations of 1.0 to 1.5 mg/ml during 1 hour stopped almost entirely the ciliary movement without permanent damage to either cells or cilia, which resumed their normal activity after elimination of the product.

Experiments with human Fallopian tube cultures pretreated with Nembutal, were then infected by incubation with a suspension containing 10^6 ID$_{50}$/ml of poliovirus. During 8-day observation no difference in response to virus infection could be found between normal and Nembutal-pretreated cultures. In other experiments tissue fragments were pretreated with enzymes to eliminate from their surface hypothetic substances which could interfere with the intimate cell-virus contact. Thus monkey trachea and human Fallopian tube fragments were submitted 30 min. at 37°C to 20 t.u./ml of bovine hyaluronidase‖ or treated similarly with 0.25% buffered (pH 7.5) solution of trypsin. The tissue fragments were cultured *in vitro,* then infected with poliovirus and observed. Hyaluronidase pretreated cultures behaved exactly as did untreated controls.

The trypsin solution acting on rabbit or human Fallopian tube fragments caused detachment of the lining sheets of ciliated epithelium, and a suspension of ciliated cells, single or in small groups, was obtained. These cells remained alive and active for at least 4 days in microdrops of medium placed on bottom of Petri dish under a 3-4 mm sheet of liquid paraffin.¶ When infected by incubation with 10^5 ID$_{50}$/ml of poliovirus suspension, and then washed with specific antipoliovirus serum, most of these trypsin pretreated cells maintained their ciliary activity as long as non-infected controls (4 days). Virus production in the microdrops containing ciliated cells could not be detected until 4th day. HeLa cells prepared and infected concurrently under same conditions, were all destroyed in <48 hrs. This was accompanied by virus production evidenced in droplets at end of this period.

Discussion. Resistance to the cytopathic action of viruses *in vitro,* observed previously on tracheal and bronchial ciliated epithelium (1), could be regarded possibly as specific defense adaptation of the respiratory tract mu-

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‖ Preparation supplied by Pasteur Inst. (Paris).

¶ These were prepared according to technic used by Dr. John Paul, Glasgow.

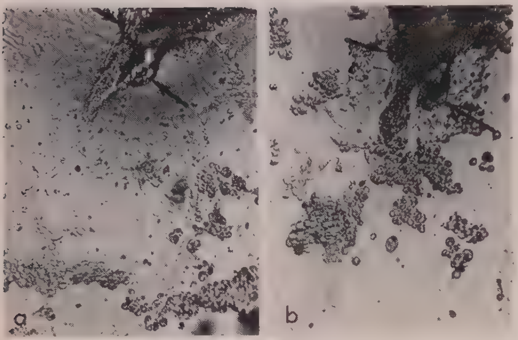

FIG. 2. 4-day-old rabbit Fallopian tube culture (a) 24 hr and (b) 48 hr after infection with herpes virus. Centripetal progression of lesions in outgrowth area and a persistent zone of ciliary activity (arrows).

cosa. However in view of above observations, this phenomenon appears as a more general one, since it can be observed as well in Fallopian tube ciliated epithelium infected with different viruses.

It must be emphasized that we are actually observing only a *relative* resistance, since even differentiated ciliated epithelium in most cases finally succumbs to virus infection. For each tissue virus combination a well defined gap exists between time of disorganized tissue destruction and attack on organized ciliated epithelium by a given virus. For I.B.R. virus and human Fallopian tube cultures f.ex., we never observed a definite action of the virus on ciliated cells, in spite of its rather strong cytopathic effect on human dedifferentiated epithelium *in vitro*. For human oviduct cultures infected with poliovirus, there were 5 to 6 days between onset of degeneration of dedifferentiated cells and cessation of ciliary activity. While in herpes infected rabbit Fallopian tube cultures the ciliated epithelium survived only 2 to 3 days longer than the nonorganized cells.

In studying response of organized tissue cultures to virus infections, Bang and Niven recently reported(4) negative results with the common cold and influenza viruses in human embryo respiratory tract mucosa cultivated *in vitro*. In a similar system adult ferret nasal mucosa and embryonic chick skin were destroyed when infected with influenza, vaccinia or herpes viruses. However these authors did not specifically compare the cytopathic effect and virus production in organized and dedifferentiated tissues.

An apparent contradiction exists between relative resistance of ciliated epithelium to virus infections *in vitro,* which we demonstrated, and observations on presence of degenerated ciliated cells in bronchial secretions of animals(5) or man(6) infected with respiratory viruses. Our observations support the evidence that at least in many instances, dedifferentiated normal or cancer cells in tissue culture, are more sensitive to virus infections than organized tissue cells even when these represent typical pathological location of a given virus infection *in vivo*. Studying relative sensitivity of brain and dedifferentiated visceral tissues of hamsters to encephalomyelitis viruses, Kissling(7) came to a similar conclusion.

One could suppose that the belated response of ciliated epithelium to action of cytopathic viruses *in vitro* is due to a period of "maturation" or metabolic transformation after explantation *in vitro* necessary for appearance of virus receptivity. It is however remarkable that the delay between introduction of virus and arrest of activity in ciliated, organized sheets of epithelium, did not seem to depend either on age of culture or on chemical or enzymatic treatment of cell surface. Another possibility is normal penetration of virus in differentiated ciliated cells followed by slowed down virus development in these cells. This might not interfere immediately with normal activity of cell organelles such as cilia. As a matter of fact poliovirus fixation and development could be demonstrated by us in pure suspension of fresh trypsinized human oviduct ciliated cells followed by virus production markedly delayed, as compared with that in similar conditions with dedifferentiated cells.

The particular behavior of ciliated epithelial cells with regard to virus infection *in vitro* can be compared with observations on peritoneal exudate cells and poliovirus(8), on human embryo heterografted skin infected with the same virus(9), on different chick cells infected with avian myeloblastosis virus(10) and many others. In general, one should not expect to find uniform pattern and timing of viral infectious processes in animal cells, even for a specific species - virus combination. With the variety of highly differentiated and specialized cells of the animal organism, one must anticipate rather a multiplicity of cell - virus reactions, much wider than that established for monocellular organisms and their viruses.

Summary. 1) Adult human and rabbit Fallopian tube fragments, which maintained in *in vitro* cultures their specific histological structure and coordinated vibratory activity, were submitted to infection with polio, adeno, bovine rhinotracheitis, herpes and other viruses. 2) The organized ciliated epithelium when compared with dedifferentiated epithelium in the same cultures showed in all cases a relative resistance to the cytopathic action of viruses, manifested by a marked delay in cell destruction, more pronounced in the case of bovine rhinotracheitis or polio and less marked in the case of herpes or vaccinia viruses. 3) This resistance could not be abolished either by immobilization of cilia with anesthetics or by treatment of cell surface with hyaluronidase or trypsin. 4) The meaning and implication of these data are discussed.

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Effect of Parathyroidectomy on Distribution of Radiocalcium. (24645)

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Tracer experiments with injected Ca^{45} have demonstrated rapid exchange between blood and bone calcium which can vary with age and with the metabolic state of bone(1,2). It has been suggested that parathyroid hormone might directly affect the amount of "exchangeable bone calcium"(3), but recent evidence indicates that the hormone can act on that portion of bone unavailable for rapid exchange(4,5). While it appears established that parathyroid hormone directly increases effective solubility of bone mineral(6), it is not certain how this effect might influence the amount of bone calcium available for rapid exchange. In the present study the effect of parathyroidectomy on the early exchange of a tracer dose of Ca^{45} in rats was examined to shed some light on this question.

Methods. Distribution of a tracer dose of Ca^{45} was studied in groups of young (70 to 82 days) and old (289 to 302 days) male Holtzman rats. In 3 groups of young rats, Ca^{45} was given at intervals of 1 day, 6 to 8 days, and 28 to 30 days after parathyroidectomy or after sham operation. In one group of old rats, Ca^{45} was given 7 days after parathyroidectomy or sham operation. Parathyroidectomy was performed by electro-cautery under nembutal anesthesia. In sham operations the parathyroids were identified but not cauterized. In each animal distribution of radioactivity was measured 6 hours after intraperitoneal injection of a tracer dose of 3 to 6 μc of Ca^{45} with less than .005 mg of carrier calcium dissolved in 0.5 ml of normal saline. Plasma radioactivity was measured in samples of tail vein blood obtained 2 and 4 hours

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* Vet. Admin. Clinical Investigator.

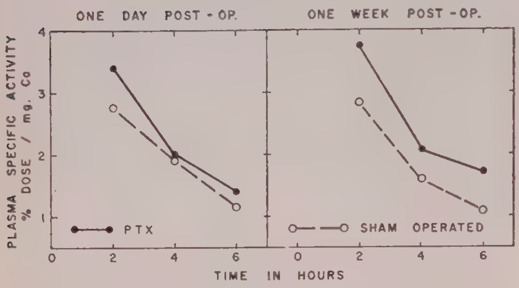

FIG. 1. Plasma specific activity after Ca^{45} inj. in parathyroidectomized and sham-operated rats. Animals studied one day after operation on left and one week after operation on right hand graph.

after injection. At 6 hours the animals were anesthetized with ether and sacrificed by heart puncture. Stomach and intestine were removed and their contents pooled with the feces (G-I sample), urine was collected during 6-hour period, and in those experiments done on young rats one month after operation, femur, heart, liver and kidney were removed for measurement of radioactivity. Serum and urine were planchetted directly, G-I and tissue samples were ashed, dissolved in HCl and then planchetted. Radioactivity was assayed in a windowless flow counter and was corrected for absorption, using a previously prepared curve. Total calcium content in serum, urine, G-I sample and tissue was determined by precipitation as oxalate and permanganate titration(7). Data are presented in terms of specific activity calculated as per cent of injected dose/mg of Ca. Following initial equilibration in the extracellular fluid space, which is probably complete in a few minutes, further changes in plasma specific activity are chiefly due to exchange with bone and tissue calcium. The amount of bone and tissue calcium which has exchanged at 6 hours can be calculated from the injected radioactivity minus the amount in G-I sample, urine, and extracellular fluid, *i.e.*, per cent of injected radioactivity in bone and tissue, divided by plasma specific activity, which is taken as equal to the specific activity of exchanging calcium. The resulting value is termed the 6-hour exchangeable calcium. It is not identical with exchangeable bone calcium but consists of several components(6) including 1) rapidly exchangeable bone calcium, 2) exchangeable calcium in muscle and skin (the amount in other organs is $<1\%$ of injected radioactivity), 3) calcium added to the skeleton as new bone. From data in the literature (2,8,9) it appears likely that the bulk of "6-hour exchangeable calcium" represents exchangeable bone calcium.

Results. The results are summarized in Fig. 1 and Table I. In young animals plasma calcium concentration decreased to about 6 mg % one day after parathyroidectomy and did not change further one week or one month after operation. While plasma specific activity was somewhat higher in parathyroidectomized animals studied one day after operation than in sham-operated controls. the difference was significant only at 6 hours ($p < .05$) but not at 2 and 4 hours. In rats studied one week after operation there was a greater difference in plasma specific activity between parathyroidectomized and control rats, significant at 2 and 4 hours ($p < .05$) and highly significant at 6 hours ($p < .001$). Moreover plasma specific activity was significantly higher in parathyroidectomized animals one week after operation than one day after operation ($p < .01$). The third group studied one month after operation showed no further increase in specific activtiy after parathyroidectomy.

Radioactivity in the G-I sample was not altered by parathyroidectomy. There was more radioactivity in urine of parathyroidectomized rats presumably because specific activity was increased while total calcium excretion was little altered. Because of the large decrease in plasma calcium concentration, the calculated extracellular fluid radioactivity at 6 hours was decreased after parathyroidectomy. The sum of extracellular, urinary, and gastro-intestinal radioactivity was similar in parathyroidectomized and sham-operated rats, so that changes in "6-hour exchangeable calcium" per rat (Table I) reflect changes in plasma specific activity. In young animals the "6-hour exchangeable calcium" was 200 to 350 mg/kg body weight, assuming the body is 2% calcium these figures represent 1 to 1.75% of total bone calcium. A value for exchangeable calcium plus any accretion that has occurred at six hours can also be obtained directly from radio-

TABLE I. Effect of Parathyroidectomy on 6-Hour Distribution of Ca^{45}.

Group			No. of rats	Age (days)	Wt (g)	Plasma Ca (mg %)	Radioactivity in ECF* (% of inj. dose)	Radioactivity in Urine (% of inj. dose)	Radioactivity in GI tract (% of inj. dose)	Plasma specific activity (% dose/mg Ca)	Exchangeable Ca† Per rat (mg)	Exchangeable Ca† Per body wt (mg/kg)
							(a)	(b)	(c)	(d)		
Young rats	One day post-op.	Sham	9	76	264 ± 10	10.0 ± .2	4.7 ± .2	1.1 ± .2	3.7 ± .2	1.14 ± .06	79	301
		PTX	10	77	252 ± 20	6.1 ± .3	3.3 ± .2	2.3 ± .1	3.9 ± .3	1.40 ± .05	65	256
	One wk post-op.	Sham	8	75	240 ± 4	10.3 ± .3	4.2 ± .3	1.5 ± .5	3.7 ± .3	1.09 ± .09	83	347
		PTX	9	76	224 ± 5	5.9 ± .3	3.5 ± .2	2.2 ± .3	3.9 ± .2	1.70 ± .06	53	238
	One mo post-op.	Sham	6	76	268 ± 5	10.3 ± .2	5.8 ± .1	1.1 ± .2	4.6 ± .3	1.34 ± .05	66	263
		PTX	7	77	251 ± 8	6.5 ± .3	4.4 ± .3	1.5 ± .1	4.1 ± .3	1.73 ± .15	52	194
Old rats	One wk post-op.	Sham	4	292	422 ± 8	10.5 ± .1	12.9 ± .2	.2 ± .1	3.8 ± .4	1.87 ± .10	44	105
		PTX	4	299	409 ± 20	7.8 ± .2	9.7 ± .2	.4 ± .1	3.9 ± .2	1.94 ± .05	44	108

Figures are mean values with stand. error.

* Calculated as: % dose/ml plasma × (plasma vol + .65 interstitial fluid vol). Plasma vol was assumed to be 4% and interstitial fluid vol 18% of body wt. Interstitial fluid [Ca] is estimated as 65% of plasma Ca and assumed to have equal specific activity.

† Calculated as: $\frac{100-(a+b+c)}{(d)}$.

activity in the femur divided by plasma specific activity. Mean values one month after operation were 1.1% of total femur calcium in parathyroidectomized rats and 1.7% of total femur calcium in sham-operated rats.

The effect of parathyroidectomy was considerably different in old rats. There was a smaller decrease in plasma calcium concentration one week after parathyroidectomy, and plasma specific activity was almost the same in sham-operated and parathyroidectomized rats. There was a much smaller urinary excretion of Ca^{45} and stable calcium. The amount of radioactivity in extracellular fluid was higher in control than in parathyroidectomized rats. This difference could account for the observed difference in specific activity since the values for exchangeable calcium were identical in parathyroidectomized and control animals. The "6-hour exchangeable calcium" was only 44 mg/rat or 100 mg/kg body weight. This represents about 0.5% of total bone calcium.

Discussion. The results suggest that parathyroid hormone does not affect the amount of bone calcium available for exchange directly. First, because there was no change in exchangeable calcium despite a decrease in serum calcium in old rats after parathyroidectomy, and second, because the change in exchangeable calcium in young rats after parathyroidectomy was not immediate but progressive, while the change in plasma calcium concentration was immediate. The progressive decrease in the "6-hour exchangeable calcium" is considered to be an indirect consequence of parathyroidectomy. If parathyroid hormone acts by direct resorption of bone then parathyroidectomy would lead to decreased bone resorption. Presumably this would result in less remodeling and less new bone formation. Since the proportion of calcium available for rapid exchange is greatest in newly formed bone(6), the exchangeable fraction would decrease as new bone formation decreased. In old rats, with less new bone formation in the controls, parathyroidectomy would be expected not to affect exchangeable calcium further.

Summary. The 6-hour distribution of a tracer dose of Ca^{45} was studied in rats at various intervals after parathyroidectomy and sham operation. In young rats studied one day after parathyroidectomy there was a marked decrease in plasma calcium and a small decrease in the "6-hour exchangeable calcium." One week after parathyroidectomy there was no further decrease in plasma calcium but a significant further decrease in 6-hour exchangeable calcium. In old rats studied one week after parathyroidectomy there was a somewhat smaller decrease in plasma calcium concentration and no change in 6-hour exchangeable calcium. It is concluded that removal of the parathyroids does not affect the amount of bone calcium available for exchange directly, but may act indirectly in the following sequence: decreased parathyroid hormone → decreased bone resorption → decreased new bone formation → decreased exchangeable calcium.

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Persistence of Heterologous Bone Marrow in Mice as Function of X-Ray Dose. (24646)

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Previous studies on recovery rate of mouse's immune mechanism after irradiation and treatment with AET† or bone marrow(1-4) led to formulation of the following hypothesis: There are many antibody-producing cells, or an antibody-producing cell, that can recognize degrees of antigenicity (homologous, closely related heterologous, and distantly related heterologous antigens); *i.e.*, degree of injury to the recognition mechanism by X-irradiation would increase in the order of mention. Makinodan and Gengozian(5,6) tested and confirmed this hypothesis on the basis of mouse's ability to produce titratable, circulating antibodies. A subsequent test would then be a biological one; *i.e.*, persistence and proliferation of foreign tissue in X-irradiated recipients as a function of genetic relation between donor and host. Our findings on such an investigation are reported here.

Materials and methods. Approximately 1100 (C3H x 101) F_1 male and female mice 12 weeks old were used as recipients. Bone marrow donors were normal male and female Sprague-Dawley rats (150-250 g), 12-week-old golden hamsters, weanling guinea pigs, and albino rabbits (1.5-2 kg). A constant-potential Phillips X-ray machine was used under following conditions: 250 kv; 15 ma; 160-170 r/min at 60 cm; inherent filtration, 1 mm of Al; added filtration, 1 mm of Al; and hvl, 0.5 mm of Cu. Mice were irradiated in circular perforated lucite container attached to revolving turntable. Generally, within 4 hr after a single exposure to 0, 300, 400, 450, 500, 600, 700, 800, 950, 1150, 1300, 1500, or 2000 r, mice in groups of 20 to 40 were given no treatment or bone marrow of rat (RBM), hamster (HBM), guinea pig (GPBM), or rabbit (RbBM). Intravenous bone marrow dose/mouse was ~ 140 x 10^6 nucleated cells from femora, tibiae, and humeri suspended in 1 ml of Tyrode's solution. After treatment, 2-10 mice/group were sacrificed on following intervals, with some exceptions: 1, 2, 3, 4, 5, 6, 16, 24 hr; 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 days. Usually, 2 blood and bone marrow smears and 15 spleen imprints from each mouse were fixed in acetone and stained for alkaline phosphatase-positive cells by Gomori technic(7). Imprints were always obtained from 3 representative areas of the spleen.

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* Operated by Union Carbide Corp. for U. S. Atomic Energy Comm.

† AET = S, 2-aminoethylisothiuronium bromide hydrobromide (fomerly called S,β-aminoethylisothiuronium bromide hydrobromide).

Results. Granulocytic cells of normal and X-rayed mice were alkaline phosphatase negative; those of donors were positive. To express relative concentration of donor cells in treated mice, we rated stained preparations 1+, 2+, 3+, and 4+ (Fig. 1). On this basis, concentration of donor cells followed essentially 3 patterns during first 10 days after injection: (a) it decreased; (b) decreased, then increased, then decreased again; and (c) decreased and then increased. Fig. 2 illustrates these patterns, which we interpreted as follows: Patterns A and A′ show elimination of HBM and RBM cells, respectively, by the unirradiated host. Presence of donor cells in an irradiated host for significantly longer time than in normal host indicates *persistence* (patterns B and B′). A secondary rise in concentration of donor cells in an irradiated host indicates proliferation or transplantation; patterns C and C′ are examples of *temporary* proliferation or transplantation of HBM and RBM cells, respectively, and patterns D and D′ are examples of *prolonged* proliferation or transplantation.

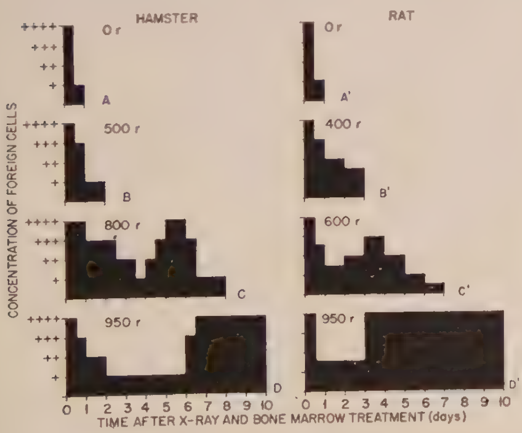

FIG. 1. Alkaline phosphatase-positive cells in spleen of mice treated with foreign bone marrow. A, 1+; B, 2+; C, 3+; D, 4+ reactions.

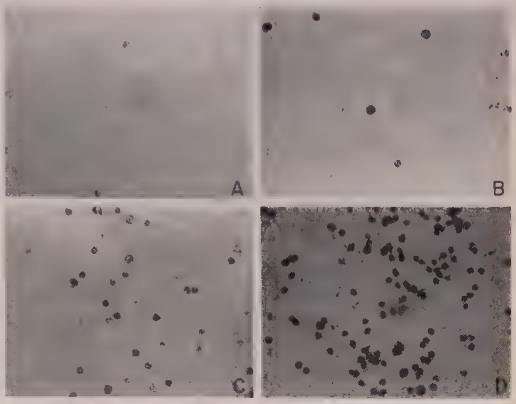

FIG. 2. Persistence, temporary and prolonged transplantation of hamster and rat bone marrow cells in mouse spleen after X-radiation and bone marrow treatment. A and A′, control: 0 r–HBM, 0 r–RBM; B and B′, persistence: 500 r–HBM, 400 r–RBM; C and C′, temporary transplantation: 800 r–HBM, 600 r–RBM; D and D′, prolonged transplantation: 950 r–HBM, 950 r–RBM.

In normal and irradiated mice treated with foreign bone marrow, large patches of intercellular alkaline phosphatase-positive material appeared in blood and bone marrow smears 2 or 3 days after treatment and persisted several days after positive cells were no longer distinguishable in these tissues. Since these patches hampered evaluation of smears, spleen imprints were generally used for determining whether positive cells were present.

Rat bone marrow injection (Table I, column 2). Donor cells were detected in blood, bone marrow, and spleen of normal mice 1 hr after injection of RBM but none were seen in blood 4 hr after treatment. Very few, if any, were found in bone marrow and spleen 1 day after treatment. With increase in X-ray dose, RBM cells persisted longer in the host—2, 3, 4, and 5 days after 300, 400, 500, and 600 r, respectively. Donor cells were present among the very few surviving 710 r-RBM mice 10

TABLE I. Persistence of Donor Cells in Mouse Spleen after Various X-ray Doses and Bone Marrow Treatment.

	Persistence time, days			
	Donor bone marrow			
X-ray dose, r	Rat	Hamster	Guinea pig	Rabbit
0	1	1	<1	<1
300	2			
400	3	1		
500	4*	1		
600	5*	5*		
700		6*		
710	>10†			
800	" ‡	8*	<1	
950	" ‡	>10‡	"	<1
1150	" ‡	> 5†	3	"
1300	> 6†	> 4†	4	1
1500	> 4†	> 3†		4
2000	> 3†			"

* Temporary transplantation.
† Short-term survivors.
‡ Prolonged transplantation.

days after treatment. The minimum X-ray dose that would permit temporary transplantation of donor cells in mouse spleen was 500 r; prolonged transplantation was evident among RBM-treated mice receiving 800 r or more.

Hamster bone marrow injection (Table I, column 3). HBM cells met the same fate as RBM in normal mice; *i.e.*, no donor cells could be detected in blood of mice 4 hr after HBM treatment or in bone marrow and spleen 1 day later. Persistence time of HBM cells among the 400- and 500-r mice was comparable to that of unirradiated mice, but was 5, 6, and 8 days after 600, 700, and 800 r, respectively. Minimum X-ray dose that permitted temporary transplantation of donor cells in mouse spleen was 600 r. Prolonged transplantation was evident among the HBM-treated mice receiving 950 r or more.

Guinea pig bone marrow injection (Table I, column 4). In normal, unirradiated mice, blood, bone marrow, and spleen contained donor cells 1 hr after injection of GPBM; donor cells were eliminated from blood in 4 hr, and from bone marrow and spleen within 18 hr. Similar elimination was observed after 800 and 950 r. However, donor cells persisted for 3 and 4 days among 1150 and 1300 r-GPBM mice, respectively. There was no evidence for even a temporary transplantation among these mice. X-ray doses higher than 1300 r were not used.

Rabbit bone marrow injection (Table I, column 5). Donor cells were rapidly eliminated from normal mice after RbBM injection; *i.e.*, blood of host became negative in 1 hr, bone marrow in 3 hr, and spleen in 6 hr. In these mice, as early as 1 hr after treatment, the spleen became congested and its weight markedly increased. Donor cells were detected in spleens of 1300 r-RbBM mice 1 day after treatment but not thereafter. However, a significantly prolonged persistence time of 4 days was seen among the 1500 and 2000 r-RbBM mice. As with the GPBM-treated mice, even a temporary transplantation was not evident among these mice.

Discussion. Of 4 heterologous bone marrows tested, RbBM was eliminated by normal adult mice slightly faster than GPBM, HBM, or RBM. Regardless of genetic relation between host and donor, however, these unirradiated adult mice eliminated foreign bone marrow within 1 day after injection. Consequently, we assumed that persistence of foreign bone marrow for 3 or more days after injection indicated an impaired immune mechanism of the host when dose, route, and injection time of bone marrow were kept constant. Increasingly large doses of X-rays (400, 600, 1150, and 1500 r, respectively) were required to permit persistence of rat, hamster, guinea pig, and rabbit cells in the mice. A higher X-ray dose was required for transplantation of HBM than RBM in the mouse. The immunogenetic experiments of Odell and Caldwell(8) showed that the minimum X-ray dose that would permit prolonged transplantation of homologous RBM in the rat was 300 r. Thus the degree of X-ray injury to the host's immune mechanism depends on the genetic relation between the host and donor; *i.e.*, a higher X-ray dose is required to impair the immune mechanism of the host against a more distantly related antigen.

GPBM may have failed to transplant in irradiated mice (950-1300 r) because the concentration of cells used (130-140 x 10^6) was insufficient; it is known that the optimum dose of RBM that will protect lethally irradiated mice is ~ 10 times that of isologous bone marrow(9-11). Lorenz and Congdon(12) and van Bekkum and Vos(11) succeeded in prolonging the survival time of a few lethally irradiated mice treated with GPBM. This would indicate at least temporary transplantation of donor cells, if it can be assumed that transplantation of donor cells is necessary for even an extension of survival time of lethally irradiated mice.

We found that (a), during maximum depression period after varying X-ray doses, mice responded beter to sheep than to rat antigens(5) and (b), recovery rate was faster for sheep antigens than for rat antigens(1,3). Published results and findings here reported substantiate our hypothesis: antibody-producing cells of normal adult mice can differentiate between and respond to closely and distantly related antigens with equal effect. The degree of X-ray injury to the recognition and response mechanisms decreases with the foreignness of the antigen.

Summary. Bone marrow from species closely and distantly related to the mouse was injected after various X-ray doses to the total body. Rat, hamster, guinea pig, and rabbit bone marrow persisted in normal, nonirradiated mice for not more than 1 day. The minimum X-ray dose that would permit persistence of rat, hamster, guinea pig, and rabbit bone marrow in mice for more than 3 days was 400, 600, 1150, and 1500 r, respectively. The minimum X-ray dose that permitted (a) temporary transplantation of rat and hamster bone marrow in mice was 500 and 600 r, respectively, and (b) prolonged transplantation of rat and hamster bone marrow in mice was 800 and 950 r, respectively. The significance of these findings is discussed.

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Effects of Immunized Parental Strain Bone Marrow on Lethally Irradiated F_1 Hybrid Mice. (24647)

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Nonirradiated bone marrow cells inoculated into irradiated recipients transplant and prevent death from hemopoietic failure. However, a syndrome called "foreign bone marrow reaction" occurs in many animals receiving antigenically foreign marrow cells after whole-body irradiation(1). Mice so afflicted seem to recover from radiation-induced injury but soon lapse into progressively wasting state, and die 30-90 days after treatment. Since implantation of adult parental spleen cells in irradiated F_1 mice hastens death if donor is pre-sensitized to recipient(2), this experiment was undertaken to determine effects of presensitized parental bone marrow on survival of irradiated F_1 hybrids.

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TABLE I. Effects of Pre-sensitization of Bone Marrow Donors on Survival of Lethally Irradiated Recipients: C3H and 101 Strain Combinations.

		Immunologic status of donor		Cumulative mortality at day*			
Recipient	Donor	Antigenic material	Wk inj. before sacrifice	30	60	90	120
(101 × C3H)F_1	(101 × C3H)F_1			2/35	2/35	2/35	2/35
(C3H × 101)F_1	(C3H × 101)F_1			0/20	1/20	1/20	
(101 × C3H)F_1	101			4/55	6/55	7/55	
Idem	C3H			0/30	0/30	0/30	2/30
(C3H × 101)F_1	101			5/61	10/61	11/61	12/61
Idem	"	(C3H × 101)F_1 tissues	1	1/39	6/39	6/39	6/39
(101 × C3H)F_1	"	C3H spleen	1	1/12	1/12	1/12	1/12
(C3H × 101)F_1	"	(C3H × 101)F_1 tissues	3	2/20	14/20	16/20	16/20
(101 × C3H)F_1	"	C3H spleen	4 or more; each wk inj.	18/28	23/28	25/28	28/28
Idem	C3H	101 spleen	*Idem*	"	"	"	
"	101	Rat "	"	0/12	0/12	0/12	0/12
"	(101 × C3H)F_1	101 "	"	0/7	0/7	0/7	0/7
"	*Idem*	C3H "	"	0/9	0/9	0/9	0/9
101	"			14/34	17/34	18/34	19/34
"	C3H			12/40	18/40	24/40	24/40
C3H	101			5/10	7/10	10/10	10/10
"	(101 × C3H)F_1			7/31	8/31	8/31	8/31

* No. dead/No. in group.

TABLE II. Effects of Pre-sensitization of Bone Marrow Donors on Survival of Lethally Irradiated Recipients: C57BL and 101 Strain Combinations.

		Immunologic status of donor		Cumulative mortality at day*			
Recipient	Donor	Antigenic material	Wk inj. before sacrifice	30	60	90	120
(C57BL × 101)F_1	(C57BL × 101)F_1			14/66	14/66	14/66	14/66
"	101			16/60	16/60	18/60	21/60
"	C57BL			31/130	68/130	106/130	115/130
"	101	C57BL spleen	1	11/25	15/25	17/25	18/25
"	C57BL	101 "	1	5/20	11/20	14/20	15/20
"	101	C57BL "	4	7/10	9/10	10/10	10/10
"	C57BL	101 "	4	3/14	11/14	13/14	14/14
101	(C57BL × 101)F_1			29/36	35/36	35/36	35/36
C57BL	"			33/34	33/34	33/34	
101	C57BL			34/40	37/40	38/40	
C57BL	101			40/40	40/40	40/40	40/40

* No. dead/No. in group.

Methods. Adult (C3H × 101)F_1, (101 × C3H)F_1, (C57BL × 101)F_1, 101, C3H, and C57BL mice were given single $LD_{100/30}$ X-ray doses of 800-900 r to the whole body. Mice were placed within separate compartments of rotating lucite drum during exposure. Physical factors were 250 kvp, 30 ma, 3 mm of Al added filtration, 0.44 mm of Cu hvl, 82-94 cm target-to-mouse distance, 90-98 r/minute. Different groups of mice received 1 femur-equivalent (about 12 million nucleated cells) of nonimmunized or pre-immunized parental strain bone marrow cells within 1 to 5 hours after irradiation. Other groups received F_1 homologous or isologous bone marrow cells (Tables I, II). Marrow cell suspensions were prepared in sterile Tyrode's solution as outlined by Congdon and Urso(3), or in sterile saline. Parental strain bone marrow donors were adults 12 weeks to 1 year old. Mice were pre-immunized by intraperitoneal inoculation of suspensions of 1) pooled (C3H × 101)F_1 thymus, liver, and spleen cells at least 3 weeks before sacrifice, 2) pooled (C3H × 101)F_1 liver, spleen, and kidney cells 1 week before sacrifice, and 3) homologous parental spleen cells 1 week before sacrifice or at weekly intervals 4 or more weeks before sacrifice (Tables I, II). For immunization controls, 101 strain mice were given weekly injections of spleen cells from adult rats for at least 4 weeks. In each instance, at least 10 million nucleated cells were inoculated/injection.

Results. Mortality 30-120 days after inoculation was low in (101 × C3H)F_1 and (C3H × 101)F_1 animals treated with isologous marrow, non-immunized parental marrow, and marrow from parental donors immunized 1 week or less before sacrifice (Table I). Conversely, survival among mice treated with marrow from donors immunized 3 or more weeks before sacrifice was poor beyond 30 days. Sensitization of donor against rat tissue did not influence mortality. Use of (101 × C3H)F_1 marrow to protect 101 or C3H parental strain recipients caused little delayed mortality. However, delayed mortality was high when 101 marrow was used to protect mice of C3H strain, and vice versa. In (C57BL × 101)F_1 mice, non-sensitized C57BL parental strain marrow gave poor survival after first 30 days (Table II). Pre-immunization of donors of either 101 or C57BL parental strains increased delayed mortality in (C57BL × 101)F_1 recipients. In parental strain mice protected with (C57BL × 101)F_1 marrow, 30-day mortality was high, as it was when marrow of one parental strain was used to protect the other.

Histological changes in F_1 hybrids sacrificed or dying after administration of pre-immunized parental bone marrow consisted principally of granulomatous proliferation of reticuloendothelial elements of the white pulp of spleen, cortex of lymph nodes, and Peyer's patches, a reaction similar to that caused by injection of parental or homologous spleen cells(2) but proceeding more slowly.

Discussion. Although administration of homologous or heterologous bone marrow is usually associated with delayed mortality in lethally irradiated recipients(1), the outcome is variable when parental strain marrow cells are transplanted into F_1 hybrids, mortality varying from strain to strain(4-7). One basis for this variability is the varying extent of histocompatibility differences between donor and recipient at the *H-2* locus(7,8). For example, in the present experiment, marrow cells from C3H or 101 donors, which are identical at the *H-2* locus ($H\text{-}2^k$) (R. D. Owen, personal communication), failed to consistently provoke delayed mortality in (101 × C3H), or reciprocal, F_1 recipients unless pre-sensitized to them. In contrast, marrow cells from C57BL donors, which differ at the *H-2* locus from 101 mice ($H\text{-}2^b$ and $H\text{-}2^k$, respectively), caused much delayed mortality in (C57BL × 101)F_1 recipients. The greater percentage of delayed mortality in (C57BL × 101)F_1 recipients injected with C57BL cells, as opposed to 101 cells, cannot be accounted for. It may conceivably denote either greater antigenicity of the F_1 to the C57BL parent than to the 101 parent by virtue of undefined differences at the *H-2* locus or other loci, or greater ability of C57BL than 101 marrow cells to initiate antibody production. Similarly, failure of nonsensitized 101 or C3H marrow cells to cause delayed death in (101 × C3H), or reciprocal, F_1 recipients cannot be explained in the face of high delayed mortality, such parental strain cells caused when transplanted into the homologous parent, unless it is assumed that the F_1 was less antigenic to parent than was the homologous parent. The latter may have been the case if antigenicity was determined by gene dose, as suggested by Uphoff(9), since the homozygous parent contained twice the gene dose of the heterozygous F_1 for any given antigen. Presumably, undefined histocompatibility differences between C3H and 101 mice at the *H-2* locus or other loci elicited the lethal reaction observed in (101 × C3H), or reciprocal, F_1 recipients when reactivity of parental strain donors was enhanced by pre-sensitization. Pre-sensitization to nonspecific (rat) antigens was without effect, as anticipated.

Enhancement of foreign bone marrow reaction by pre-sensitization of donor against the recipient is notable in that it denotes heightening of antibody-forming potentiality of injected bone marrow cells. The effect is the reverse of that obtained by transplantation of fetal hemopoietic tissue(10). It is also noteworthy that pre-sensitization did not increase the marrow's lethality to the level of spleen or other lymphoid tissues(2,11-14) or exert any detectable effect unless carried out well in advance of transplantation.

If the foreign bone marrow reaction is similar to foreign spleen reaction in pathogenesis, as suggested by histopathologic resemblances, it could be initiated by lymphoid cells in bone marrow. In this case, differences between effectiveness of spleen and marrow would represent merely numerical differences in numbers of reactive, antibody-forming cells present in each tissue. On the other hand, the characteristically different time sequences of the 2 reactions suggest the differences between effects of marrow and spleen result from qualitative dissimilarities, yet to be elucidated. Since antibody-forming cells, not normally detectable in bone marrow, may be found there in highly immunized animals(10), the effects of pre-sensitization may conceivably represent release of such cells from spleen and lymph nodes and their migration to other sites, including the marrow.

Summary. Pre-sensitization of parental strain donor mice against F_1 hybrid recipients accentuated the foreign bone marrow reaction caused by injection of parental marrow cells into lethally irradiated F_1 recipients. This effect occurred with use of parental strains (101 and C3H) that were histocompatible at the *H-2* locus, as well as with those (C57BL and 101) that were histoincompatible at this locus. These results are interpreted to indicate the importance of relative antibody-forming potency of implanted cells, as well as the extent of histocompatibility differences in pathogenesis of the homograft reaction.

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Adenylic Acid Deaminase Activity in Spectrum of Rat Tumors and in Normal Liver.* (24648)

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Deamination of adenosine-5′-monophosphate (AMP) to inosine-5′-monophosphate (IMP) is catalyzed by the enzyme 5′-adenylic acid deaminase. This enzyme was demonstrated in skeletal muscle and in water-soluble fraction of smooth muscle, liver and kidney. Its isolation in crystalline form from rabbit skeletal muscle was recently reported. The above work was summarized by Lee(1). Greenstein *et al.*(2) observed deamination of yeast adenylic acid (presumably adenosine-3′-monophosphate) by several normal tissues of rat and mouse and by a mouse hepatoma. Recently Fodor *et al.*(3) showed that homogenates of both growing and regressing Flexner-Jobling carcinomas deaminated adenosine-5′-monophosphate, while their activity towards the 3′-isomer was much lower. Our purpose was to determine the 5′-adenylic acid deaminase activity in a number of rat transplantable tumors, and to ascertain whether activity in hepatomas resulting from dye carcinogenesis

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TABLE I. Adenylic Acid Deaminase Activity in Tumor Tissue Homogenates.

Tissues	Enzymatic activities* Homogenates	Enzymatic activities* Cell suspension homogenates†
Skeletal muscle	(1)‡ 32.6	
Walker carcinosarcoma 256	(2) 7.78 ± .92§	(4) 2.78 ± .08
Jensen sarcoma	(4) 6.92 ± .80	(2) 2.47 ± .13
Flexner-Jobling carcinoma	(2) 4.38 ± .62	
Novikoff hepatoma	(7) 1.12 ± .05	

Experimental conditions described in methods section.

* Enzymatic unit: μM of ammonia produced/mg homogenate N/15 min.

† Homogenates from cell suspensions; see text, method section.

‡ No. of observations in parentheses.

§ Stand. dev.

was altered from that observed in normal liver.

Materials and methods. Female Holtzman rats were used. Jensen sarcoma and Walker carcinosarcoma 256 were carried intramuscularly using the transplantation method described by Talalay *et al.*(4). The Flexner-Jobling carcinoma was carried subcutaneously, while the Novikoff hepatoma and a hepatoma (MDAB) induced by feeding 3′-methyl-4-dimethylaminoazobenzene were carried intraperitoneally. At appropriate intervals the tumors were harvested and freed of gross necrotic or hemorrhagic areas. In homogenate studies the tumor tissues were stored at −25°C not exceeding 2 days. When homogenates were prepared from cell suspensions, freshly excised tumor particles were fragmented with loose-fitting glass tissue suspender in phosphate buffer (0.039 M, pH 7.4) containing 0.25 M sucrose. Cells were separated from the debris by 3 successive passages through glass-wool and filtrates were centrifuged at 100 g for 2 minutes. The supernatant was removed by suction and the cells washed once with citrate buffer (0.05 M, pH 6.5). Pathological examination of suspensions showed them to be essentially free of debris and to be contaminated with inflammatory cells to 4% by cell count.† Since deamination products of AMP were IMP and ammonia(1), ammonia production, determined by the method of Boyce(5), was used to follow extent of the reaction. Tissues or cell suspensions were homogenized in citrate buffer (0.05 M, pH 6.5) and pre-incubated 10 minutes at 38°C. Homogenates were then added to incubation mixture which consisted of 0.05 M citrate buffer (pH 6.5) and 0.006 M AMP‡ in total volume of 15 ml. Aliquots for anaysis were removed at appropriate time intervals and total incubation was 15 minutes. A linear relationship between enzymatic activity and time of incubation was obtained. Controls, in which either AMP or homogenates were omitted from the reaction mixture, were determined simultaneously in each experiment and only negligible ammonia production was observed. The unit of enzymatic activity is expressed as μM of ammonia produced/mg homogenate nitrogen/15 minutes incubation. Since inorganic phosphate concentration in the reaction mixture remained constant during the incubation period, the ammonia assayed apparently was derived directly from AMP and was not mediated by a dephosphorylation reaction.

Results. All tumor tissues studied deaminated AMP and data summarized in Table I. Homogenates of Walker and Jensen tumors exhibited highest activity among all neoplasms examined, but their activity was approximately 30% that of skeletal muscle. Since both tumors were transplanted intramuscularly, it was possible that the homogenates may have been contaminated with muscle tissues. Therefore, cell suspensions of Walker and Jensen tumors were prepared and homogenized. Under these conditions, the deaminase activity approached the level observed in Flexner-Jobling and Novikoff tumors. Thus, a portion of activity which was observed in crude homogenates of Walker and Jensen

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† The authors wish to express appreciation to Dr. E. S. Irvine for pathological examinations.

‡ Pabst Labs.

TABLE II. Comparison of Adenylic Acid Deaminase Activity in Homogenates of Normal Liver, Primary Hepatoma, and Transplantable Hepatomas.

Tissue	Enzymatic activities*	
Liver	(4)†	.69 ± .07‡
MDAB primary hepatoma	(4)	.88 ± .04
MDAB transplanted hepatoma§	(3)	3.00 ± .09
Novikoff hepatoma	(7)	1.12 ± .05

Experimental conditions described in methods section.

* Enzymatic unit: See Table I.

† No. of observations.

‡ Stand. dev.

§ This tumor has been carried for 52 transplant generations.

tumors may have been contributed by muscle or other nonneoplastic tissues.

Since Greenstein(2) reported that mouse hepatomas deaminated yeast and thymus nucleic acids to a greater extent than normal livers, the adenylic acid deaminase activity in primary and transplanted hepatomas and normal livers was compared (Table II). Activity in the 2 transplantable hepatomas (Novikoff and MDAB) was significantly higher ($P < .01$)[§] than normal liver; however, activity in a primary hepatoma (MDAB primary hepatoma) was equivalent to that observed in normal liver. In other studies in which hepatoma and liver were compared(6,7), the activities of several purine-metabolizing enzymes (5′-nucleotidase, nucleoside phosphorylase, guanase, adenase, xanthine oxidase and uricase) were markedly reduced, while adenosine deaminase activity was unchanged(6) or increased(7).

Our data suggested at least 2 explanations concerning the effect of azo dye carcinogenesis upon adenylic acid deaminase activity of liver. (a) Deaminase activity of liver was not altered during carcinogenesis but subsequent transplantation of primary hepatomas resulted in significant elevation; (b) Since it has been reported that more than one kind of tumor may be induced by azo dyes(8), the 3 hepatomas studied (Table II) may have been different neoplasms.

Summary. 5′-adenylic acid deaminase activity of a spectrum of transplantable rat tumor homogenates was determined, and all tumors studied possessed deaminase activity. Activity in the primary hepatoma induced by 3′-methyl - 4 - dimethylaminoazobenzene was equivalent to that of normal liver, while the Novikoff hepatoma and a transplanted hepatoma (MDAB) had significantly higher deaminase activity than either the primary hepatoma or normal liver.

[§] Group comparison "t" test.

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Serum Spheroplasts of *Shigella dysenteriae*. (24649)

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We reported(1) on isolation of strains of *Shigella dysenteriae* with inherently different resistance to the bactericidal effects of "normal" human serum and on the inverse correlation between resistance to serum and resistance to penicillin. In view of the latter relationship, which agrees with the assumption that both penicillin and serum affect synthesis and/or integrity of cell-wall components(*cf.*, 2,3), an effort was made to de-

TABLE I. Survival of *Shigella dysenteriae*, Strain S_3, Organisms following Exposure to Serum (Dilution 1:3) and Penicillin (2,000 U/ml) in Absence and Presence of 20% Sucrose.

	Viable count at:		
	0	30	60 min.
Treatment with:	(colonies/0.1 ml)		
Serum	426	255	60
" + sucrose + Mg^{++}, Ca^{++}	418	372	356
Penicillin	466	76	6
" + sucrose + Mg^{++}, Ca^{++}	397	380	360
" + serum	414	46	12
" + " + sucrose + Mg^{++}, Ca^{++}	427	71	20

termine whether, as in the case of penicillin (4), a hypertonic medium might interfere with the bactericidal effects of serum. With penicillin and certain other wall-damaging agents(5), it is now well established that a hypertonic medium, such as 20% sucrose, is capable of protecting sensitive bacteria from lysis by these agents, because the hypertonic environment can assume the "corsetting" functions normally provided by the intact bacterial cell wall. The resulting viable spheric structures have been referred to as *protoplasts* when essentially all wall material has been removed, as in the case of lysozyme-treated gram-positive bacteria, or as *spheroplasts** when the wall-deficiency was incomplete, as in the case of penicillin- or glycine-treated gram-negative organisms(*cf.*, 5). The present report confirms prior observations on the protective effects of sucrose on serum lysis(6) and demonstrates that the resulting viable structures represent unique spheroplasts. Somewhat similar observations have been made independently but simultaneously by Muschel *et al.*(7).

Methods. Strains S_1 and S_3 of *Sh. dystenteriae*(1) were employed. The bacteria were grown on tryptose agar for 18-24 hours at 37° C and exposed to bactericidal concentrations of serum (usually obtained from one human donor) either in tryptose broth or ABA buffer (8) supplemented with 0.05% $CaCl_2$, 0.05% $MgCl_2$, and various concentrations of carbohydrates. Prior to use, all serum samples were stored at –40°C. Depending upon serum susceptibility of the bacterial strain, final serum concentration was either 1:2.5 or 1:6. After various periods of exposure at 37°C, samples of the bacterial suspensions were plated, in appropriate dilutions, on tryptose agar. Aliquots of the bacterial suspensions were also examined microscopically under the phase microscope; an alternate technique for microscopic studies of cell structures employed fixation with 1% formaldehyde and subsequent staining with methylene blue.

Results. Addition of 20% sucrose to usually bactericidal serum dilutions resulted in complete protection, *i.e.*, survival, of the bacteria. A similar protection was not produced by lactose, starch, galactose, or glucose, added in 10 or 20% concentrations. Microscopic observations on the viable elements in serum-sucrose suspensions supported the assumption that sucrose protection involved a prevention of lysis of wall-deficient cells; spheroplasts were indeed formed under the influence of normal serum in the presence of 20% sucrose. Fig. 1a shows the appearance of untreated *Shigella* S_3 cells and Fig. 1b shows typical protoplast-like spherical cell structures obtained after 3 hours of treatment. The illustrated material is from stained preparations but comparable observations were also made on living cells, both for *Sh. dysenteriae* and *E. coli* strains, with the aid of phase microscopy. Thus, normal serum, in the presence of an appropriate, hypertonic environment, can lead to spheroplast formation of gram-negative bacteria comparable to that reported in cases of treatment with penicillin, lyso-

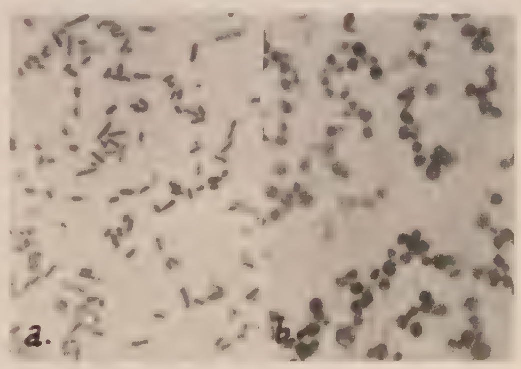

FIG. 1. *Shigella dysenteriae*, fixed with 1% formaldehyde and stained with methylene blue, before (a) and after (b) treatment with normal human serum in the presence of 20% sucrose.

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* A term proposed by K. McQuillen.

zyme, glycine, and certain other agents(5) affecting bacterial cell walls. The failure of lactose and certain other carbohydrates to produce, in the presence of serum, the required protection against lysis of *Sh. dysenteriae* may be ascribed to the cells' possession of permease systems for these sugars. Heat-inactivated (56°C for 30 minutes) sera were found to lack the capacity for spheroplast formation in the presence of sucrose; this heat inactivation paralleled the inactivation of bactericidal factors, indicating involvement of heat-sensitive complement in both reactions. Rate of spheroplast formation by serum + sucrose depended upon serum concentration and was proportional to the inherent level of serum sensitivity of the strain used. Prior absorption of the "normal" sera with *Sh. dysenteriae* organisms significantly reduced both bactericidal and spheroplasting activities, whereas *E. coli* absorption did not, which suggests that the blood of the "normal" donors may actually contain considerable quantities of specific antibodies.

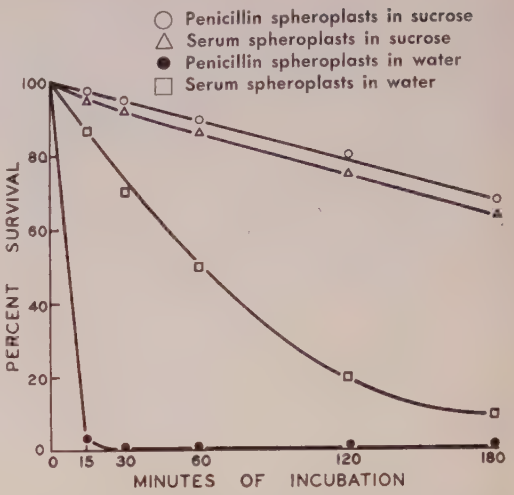

FIG. 2. Comparison of stability of penicillin-induced and serum-induced spheroplasts of *Shigella dysenteriae*.

Optimal conditions for obtaining serum spheroplasts differed considerably from those reported for penicillin protoplast and spheroplast formation. In the case of penicillin spheroplasts, cells taken from the exponential phase of growth have been reported to be most suitable; in contrast, for serum spheroplast formation, bacteria obtained from the stationary phase (18-24-hour-old broth cultures) appear to be best. Furthermore, growth is generally required for formation of protoplasts or spheroplasts by penicillin in the presence of sucrose; in contrast, serum spheroplast formation occurred with high efficiency even when serum + sucrose was added to saline suspensions of cells taken from the stationary phase of broth cultures (simultaneous control cultures confirmed that penicillin failed to affect such cells). The latter observation suggests that serum lysis may be, at least partially, enzymatic in nature. The site of attack in the cell wall also appears to be significantly different in the case of penicillin and serum. This is indicated by the fact (Table I) that 20% sucrose is capable of exerting almost complete protection against the bactericidal effects of either serum alone or penicillin alone, but fails to protect against the combined effects of serum plus penicillin. The latter failure may be explained on the assumption that the combination of serum plus penicillin may produce so many lesions in the cell wall that even the corsetting capabilities of 20% sucrose are insufficient to protect against lysis. However, it may be added that after formation of spheroplasts by exposure to penicillin plus sucrose, such spheroplasts prove resistant to the effects of serum, and, of course, serum spheroplasts, maintained in a non-growing condition in serum plus sucrose fail to be affected by penicillin.

Finally, serum spheroplasts displayed considerably greater resistance than penicillin spheroplasts to osmotic shock produced by resuspending previously formed spheroplasts in large amounts of distilled water (Fig. 2). This differential resistance again is highly indicative of significant differences in the remaining wall structures of penicillin and serum spheroplasts.

Summary. Exposure of *Shigella dysenteriae* organisms to bactericidal "normal" human sera in the presence of 20% sucrose results in survival of the bacteria and formation of wall-deficient spheroplasts. These "serum spheroplasts" differ from "penicillin spheroplasts" in several respects, including their susceptibility to osmotic shock. The relation of these observations to certain aspects of the

phenomenon of bacterial lysis by serum has been discussed.

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Echo Virus Type 13. I. Isolation and Characteristics.*† (24650)

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The first 13 ECHO prototype viruses were described in Dec. 1955 by the ECHO Virus Committee(1). Prototype ECHO-13, Hamphill 2-188-20‡, had been isolated in 1954(2) from rectal swab taken Nov. 30, 1953 from a healthy 21-month-old son of American military family stationed at Clark Air Force Base in the Philippines. During 8 weeks preceding collection of above specimen, this infant yielded poliovirus type III and ECHO type 1. Antibody responses occurred to all 3 viruses. Prior to acceptance as prototype ECHO-13, 2-188-20 virus was reciprocally tested against relatively low-titered antisera for the 12 other ECHO prototype viruses by our laboratory and those of Melnick and Sabin. No cross neutralization occurred. The above tests were conducted with 4th passage of 2-188-20 virus in rhesus kidney cell cultures; the $TCID_{50}$ titer was approximately $10^{-4.0}$/0.1 ml. After 3 additional passages the titer rose to $10^{-7.0}$. Aliquots of passage 7 were sent to Dr. Herbert A. Wenner and Microbiological Associates for preparation of hyperimmune monkey and rabbit serum respectively. Composite neutralization tests with these antisera have been reported(3). These data indicated that there was a cross relationship between ECHO viruses 1, 8 and 13 or that passage 7 of 2-188-20 contained either ECHO-1 or 8 and ECHO-13 viruses. A thorough study was therefore undertaken and pure ECHO-13 prototype strain sought. The findings are reported herein. Epidemiologic and clinical studies on ECHO-13 infections are to be published separately.

Materials and methods. Monolayer cell cultures of trypsinized rhesus monkey kidney (MKCC) were used in isolation, neutralization tests, preparation of virus pools and plaque studies. Virus pools were grown in medium 199 without serum. Neutralization tests were performed in 0.5% lactalbumin hydrolysate in Earle's balanced salt solution containing 0.5% calf serum. All media contained 400 units of penicillin, 0.2 mg of streptomycin, 20 units of polymyxin and 25 units of mycostatin/ml. Neutralizing antibody (NA) and complement fixation (CF) tests were performed as described previously(4). Results of NA tests were expressed as reciprocal of serum dilution calculated by Reed-Muench method(5) to protect 50% of inoculated cell cultures. Monkeys were hyperimmunized by repeated intramuscular injection of emulsion containing equal volumes of Ar-

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† Technical assistance of Della M. Whitaker is gratefully acknowledged.

‡ 2-188 is number given this child in field study and 20 is number of specimen from which virus was isolated.

TABLE I. Cross Neutralization Tests with 2-188-20 Derivatives, Strain 11-4-1 and ECHO Types 1 and 8.

	Virus and $TCID_{50}$					
Monkey antiserum	ECHO-1 600–32*	ECHO-8 200–64	2-188 (1 + 13) 500–20	2-188 (As-1) 320–50	2-188 (R pl) 200–32	11-4 (pl) 1,000–100
ECHO-1 (Wenner)	*3,200–27,000*	320–2,000	<8–10,000	<10	<10	<10
ECHO-8 (Wenner)	125–1,300	*5,500–20,000*	<8–630	"	"	"
ECHO-"13" (2-188 (1 + 13))† (Wenner)	50–2,000	10–160	*640–20,000*	1,280–2,560	640	40
2-188 (As-1)‡	<10	<10	<10	*640–2,560*	"	320
2-188 (R pl)§	"	"	"	20,000–40,000	*20,000–>40,000*	5,120–40,000
11-4 (pl)‖	"	"	"	20,000–>40,000	10,000–>40,000	*10,000–>40,000*

* $TCID_{50}$ = Max and min as determined by control titrations of several tests, yielding range of serum titers shown below as reciprocals of dilutions giving 50% neutralization.

† ECHO-"13" (2-188 (1 + 13)) = Original ECHO-13, passage 7, considered a mixture of ECHO-1 and ECHO-13.

‡ 2-188 (As-1) = 2-188 (1 + 13) after 6 passages in 1:5 dilution of Wenner ECHO-1 antiserum.

§ 2-188 (R pl) = Virus pool from plaque (pl) of reisolation (R) of 2-188-20.

‖ 11-4 (pl) = Virus pool from plaque of 11-4-1 strain of ECHO-13.

lacel-Bayol-F adjuvant and unconcentrated or concentrated virus-containing cell culture fluids. Concentrated virus suspensions, when used, were lyophilized and reconstituted to 1/20 of original volume. Prior to lyophilization they were extracted with fluorocarbon by the method of Manson *et al.*(6); these procedures apparently did not alter viral antigenicity. Without concentration, even after repeated injections, a highly potent immune serum was not readily obtained. The plaque technique of Hsiung and Melnick(7) was employed.

Purification of ECHO-13 virus by neutralization with appropriate antibody. Undiluted 2-188-20 virus (hereinafter referred to as 2-188(1 + 13) to denote possible mixture of ECHO-1 and 13) was passed 6 times in MKCC in the presence of 1:4 dilution of Wenner ECHO-1 monkey antiserum. The virus thus obtained, referred to as 2-188(As-1) to denote passage with ECHO-1 antiserum, was not neutralized by ECHO-1 and 8 antisera; it was, however, neutralized by Wenner ECHO-13 antiserum (Table I). Similar results (not shown in Table I) were obtained with a virus, 2-188 (As-8), separated in the same manner using ECHO-8 antiserum. Both 2-188(As-1) and (As-8) viruses were then passed 10 times in absence of ECHO-1 or -8 antisera and retested; there was no change in above neutralization pattern.

Results of cross-neutralization tests with an antiserum prepared to 2-188(As-1) virus are shown in Table I; the results with 2-188(As-8) antiserum (omitted from Table) were essentially identical. A 1:10 dilution of 2-188-(As-1) antiserum, with a homologous titer of 1:640 to 1:2,560, did not neutralize approximately 100 $TCID_{50}$ of ECHO viruses 1, 8 and 2-188 (1 + 13). By using the 2-188(As-1) antiserum to neutralize the "ECHO-13" component a second virus was found; it was clearly identified in NA tests as an ECHO-1 virus. The ECHO-1 component was also obtained from 2-188(1 + 13) by terminal-dilution and by plaque isolation.

Establishment of pure ECHO-13 virus. Reisolation of 2-188-20 virus. Since 2-188 (As-1) and (As-8) strains were "derived" by use of antisera, it was necessary to establish that ECHO-13 occurred in nature as a distinct virus. One approach was to reisolate 2-188-20 from the original rectal swab eluate which had been stored at –20° to –30°C for nearly 4 years after swab had been collected.

A virus was isolated and given 10 passages in MKCC. The 10th passage was tested against appropriate battery of antisera and was neutralized only by 2-188(As-1), 2-188(As-8) and Wenner ECHO-13 antisera. Two monkeys were hyperimmunized with a MKCC pool of a plaque isolate, 2-188(R pl), from the reisolated 2-188-20 virus. The results obtained with this antiserum were similar to those obtained with 2-188(As-1) and 2-188 (As-8) antisera (Table I).

Isolation of other strains of ECHO-13 and selection of a new prototype strain, Del Carmen 11-4-1. When we undertook this study concerning relationship of ECHO-1, 8 and 13 viruses, we had identified 3 other ECHO-13 strains(2) using a low-titered rabbit antiserum prepared to passage 4 of the original 2-188-20 virus. However, these strains were neutralized only by a 1:20 or 1:40 dilution of Wenner ECHO-13 antiserum. The Del Carmen 11-4-1 strain (referred to subsequently as 11-4) had been isolated in April, 1955 from rectal swab obtained August 6, 1953 from a healthy 4-month-old female Filipino. An apparently identical virus was isolated from throat swab collected at the same time. A virus pool of a plaque isolate, 11-4(pl), was prepared from the fecal isolate and 2 monkeys hyperimmunized. This antiserum neutralized 2-188(As-1) virus to essentially the same titer as that of the homologous agent (Table I); it did not neutralize ECHO-1 and 8 viruses.

The 2-188(R pl) and 11-4(pl) antisera were prepared simultaneously, using the same 5-month immunization schedule. The final 2 virus injections, which were given essentially 2 and 4 weeks prior to final bleeding, contained 20-fold concentrated virus. Homologous titers of resultant antisera varied from 1:10,000 to >1:40,000 (Table I). Testing of the other pure ECHO-13 strains (only 2-188(As-1) shown in Table) revealed essentially equivalent titers with 11-4(pl) serum. However, 2-188(R pl) serum titers were about 4-fold lower against 11-4(pl) virus and others than against the homologous strain. These differences were readily confirmed. Similarly, the ECHO-13 (Wenner) antiserum prepared from 2-188(1 + 13), although neutralizing approximately 100 $TCID_{50}$ of 2-188 (R pl) and 2-188(As-1) in a 1:640 to a 1:2,560 dilution, only neutralized a similar amount of 11-4(pl) virus in a 1:40 dilution (Table I). As pointed out above, 2 other strains of ECHO-13 were also neutralized by Wenner ECHO-13 antiserum but only at this same low dilution.

Both purified ECHO-13 viruses and their respective antisera were then tested reciprocally with all other enteroviruses known to propagate in MKCC. No significant heterologous neutralization occurred in either direction.

General characteristics of ECHO-13 virus. ECHO-13 strains, 2-188(R pl) and 11-4(pl) were non-pathogenic for 1-day-old suckling mice even after serial blind passage. Likewise, they were not pathogenic for 7-day-old chick embryos inoculated by yolk sac route. Both strains failed to produce apparent illness in several rhesus monkeys inoculated by either intracerebral or intramuscular routes; similar results were obtained when 11-4(pl) was inoculated intracerebrally and intraspinally into 2 monkeys. These latter 2 monkeys were sacrificed after 21 days. A chronic meningoencephalitis was observed in sections of brain stem and mid-brain but not in the cord. These findings will be reported later.

Both strains produce small and irregular plaques in MKCC bottle preparations. Cytopathogenic effect (CPE) produced in MKCC roller tubes cannot be differentiated morphologically from that produced by polioviruses and most other enteroviruses. On primary isolation of strain 11-4, CPE first appeared on 7th day. After 2 passages CPE became quite marked by 4th day. Strain 11-4(pl), at 8th passage, produced initial CPE in 24 to 48 hours and proceeded to completion by 5th day with a titer of $10^{-6.7}/0.1$ ml. Strain 2-188(R pl) behaved in similar manner. Replication of ECHO-13 virus with CPE has been observed in H.Ep. #2 and Detroit 504 Fb-L cell lines(8). Variable results have been obtained with HeLa cells, while primary cell cultures of hamster and gerbil kidney§ and monkey heart cells(9) have given negative results.

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§ Tests with gerbil kidney cells were performed by G. M. Hodges and M. B. Dobkin of Dr. F. S. Cheever's laboratory in this Department.

Both 2-188(R pl) and 11-4(pl) strains grown in MKCC were tested for ability to hemagglutinate chick and human group O red blood cells at 4°C, 25°C and 37°C. Chick cells were not agglutinated, but human cells were agglutinated equally well at 4°C and 25°C, but not at 37°C. Strain 2-188 (R pl) titered 1:64 while strain 11-4(pl) titered 1:8.

Infected MKCC fluids can be used in CF, NA and hemagglutination inhibition (HAI) tests for viral identification with hyperimmune sera and for detection of antibodies in human sera.

Discussion. It has been demonstrated that passage of Hamphill 2-188-20, which was originally distributed as ECHO-13 prototype virus, contained a mixture of ECHO-1 and 13 viruses. Whether the original rectal swab contained both viruses could not be established. After approximately 4 years of storage the rectal swab suspension from which ECHO-13 was originally isolated, was retested; only ECHO-13 virus could be reisolated. The child from whom the isolation was made had been infected with ECHO-1 just prior to or concurrently with ECHO-13 infection as demonstrated by ECHO-1 virus isolation and serological response. However, when the original ECHO-13 isolation was effected many isolations of ECHO-1 were also being made and were being handled extensively in the laboratory. If both viruses were contained in the rectal swab originally, or even if ECHO-1 was added inadvertently during laboratory passage, in retrospect it appears that in early passages only a small amount of ECHO-1 was present. Only in later passages as ECHO-1 began to outgrow ECHO-13 was its presence detectable. Further passages increased ECHO-1 and decreased ECHO-13. The fact that ECHO-1 propagates more rapidly and generally attains a higher titer than does ECHO-13 supports these contentions.

Presence of ECHO-13 was demonstrated with certainty only when the ECHO-1 component was suppressed with neutralizing antibody. The only objection to separation of viruses by this latter method is hypothetical, in that antibody in some unknown manner might alter the antigenic properties of the propagating virus, as seen *in vivo* in mice actively immunized with influenza A virus (10). Although this possibility was not investigated extensively, the antigenic properties of "antiserum-derived" ECHO-13 viruses, 2-188(As-1) and (As-8), were not observed to differ from subsequently reisolated 2-188 (R pl) strain.

Two plaque purified strains of ECHO-13, 2-188(R pl) and 11-4(pl), have been studied to determine which would be the better prototype virus. Both fulfill requirements of an ECHO virus as described by ECHO Virus Committee(3). Their antigenic potentials when used to hyperimmunize monkeys are essentially equal. However, results with test bleeding from each of 2 monkeys immunized with each virus taken every 2 or 3 weeks during immunization, suggested that 11-4(pl) antisera were broader than 2-188(R pl) antisera. Even after the 2-188(R pl) monkeys were given concentrated virus, their antisera still neutralized other ECHO-13 strains to a slightly lesser degree than the homologous strain. It should be mentioned parenthetically that perhaps this immunization technic should be employed to prepare hyperimmune antisera to those enteroviruses, such as Coxsackie B's, which exhibit considerable variation in neutralization capacities(11). Because of apparently broader antigenic pattern of 11-4(pl) and the fact that it propagates in cell culture more rapidly and generally with a higher infectivity titer, $10^{-6.7}$ compared to $10^{-5.5}$ for 2-188(R pl), 11-4(pl) was recommended to and accepted by the Enterovirus Committee as the new ECHO-13 prototype. On the basis of our total work to date there is no convincing evidence that either strain serves to demonstrate presence of antibody in an unknown serum better than the other. However, since 11-4(pl) has a higher infectivity titer and, therefore, can be used in higher dilution, it should serve as the virus of choice for ECHO-13 NA tests.

Although no grouping of ECHO viruses has been accepted by the Enterovirus Committee, ECHO-13, as determined by plaque morphology, falls into the proposed group A of Hsiung and Melnick which contains types 1, 2, 3, 4, 5, 6, 6′, 9, 11, 13 and 14(12). Furthermore, ability of ECHO-13 to hemagglutinate human group O erythrocytes at 4°C and 25°C, but not at 37°C, is characteristic of ECHO types 3, 6 and 11(13); as noted above these latter 3 virus types are included in Hsiung and Melnick's proposed group A.

Summary. 1) The originally distributed ECHO-13 prototype virus, Hamphill 2-188-20 passage 7, is a mixture of ECHO virus types 1 and 13. ECHO-1 was found in higher titer than ECHO-13 during passage and only the former was isolated at terminal dilution. It was necessary to neutralize ECHO-1 virus with antiserum to demonstrate the presence of ECHO-13 in this mixture and this apparently did not alter its antigenic properties. ECHO-13 virus was successfully reisolated in pure form from the original rectal swab eluate after 4 years storage. 2) Plaque purified strains of reisolated 2-188-20 virus and a Del Carmen 11-4-1 virus isolate were examined as possible ECHO-13 prototypes. The plaque purified 11-4-1 strain produced a broader spectrum antiserum in monkeys in early test bleedings and produced a higher infectivity titer in MKCC. It has been accepted as prototype ECHO-13 by the Committee on Enteroviruses. 3) The biologic properties of ECHO-13 virus in certain animals, chick embryos, cell culture and cell culture fluids are described. A clinically inapparent meningoencephalitis occurred in inoculated monkeys.

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Identification of a Prolonged Post-synaptic Potential of Cerebral Cortex.*

(24651)

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Our prior observations upon cerebral cortex using d.c. recording(1,2) indicate that a long-lasting surface negativity (150-300 msec. duration) develops in the wake of the evoked dendritic potential of negative polarity described by others(3,4). Chang(4) likewise observed that a negative wave could follow the dendritic potential evoked by a single stimulus. Following one surface stimulus the slow negativity we are to describe was observed to be partially submerged by a simultaneously occurring slow positivity (positive after-effect), the signs of the 2 potentials giving the appearance of summing algebraically. However, with serial stimulation (6-20/sec.) the slow positivity disappears uncovering the slow negativity which then increases in amplitude through summation. With such serial stimulation the slow negative responses to the successive shocks also fuse to provide a smooth d.c. change which can outlast a one sec. stimulus

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period by 1-3 sec. Formerly we called this slow negativity a negative after-effect and viewed it as resulting from a repolarization of dendritic elements as they recover from the preceding excitation (*i.e.*, from the dendritic potential, 1). Thus we linked the slow negativity with the dendritic potential as a sequent in the excitation-recovery cycle. However, further investigation indicates that the late wave is an independent excitation process separate from that of the primary potential. This conclusion is based upon threshold differences between primary potential and slow negativity, upon differences in the vertical distribution of the 2 potentials in the cortex as they are activated together by a sufficiently strong surface cortical stimulus, and upon their different sensitivities to certain drugs.

Method. The studies reported were done upon rabbit's frontal cortex, using 25 animals. Cat's anterior suprasylvian cortex gave corroborative results. The animals were prepared under ether anesthesia with procainization of wound surfaces. Thereafter, flaxedil and artificial respiration were substituted. Blood pressure and pulse were monitored continuously with a Sanborn electromanometer. Calomel electrodes and a d.c. amplifier and oscillograph permitted undistorted recording of the slower potentials. Monopolar recording was employed placing a critical electrode upon the cortex and a reference one upon the frontal periosteum. The stimulating electrode was a tripolar one placed within 0.5 mm of the critical recording one and composed of the points of 3 insulated 100 μ wires arranged to form an equidistant triangle. Tip separations were 75 μ. These wires were led to the stimulator through a circuit(5) which controlled shock artifact. A Grass stimulator was employed, the stimulus being 3-15 V. at 0.01 msec. duration.

Results. Under these experimental conditions a single threshold stimulus evoked the primary negative potential followed by a surface-positive after-effect. That after-effect grows in amplitude with growth of the dendritic potential as stimulus strength is increased from 3-10 V. (Fig. 1 *A*). Ordinarily at 10 V., but variable from one animal to another, the surface positivity commences to be submerged by a rapidly developing slow negativity. The stimulus necessary to elicit the latter is maximal or supramaximal for the preceding dendritic potential (compare traces, Fig. 1 *A*, 12 and 15 V.). This is the slow negativity which we have observed to summate upon serial stimulation.

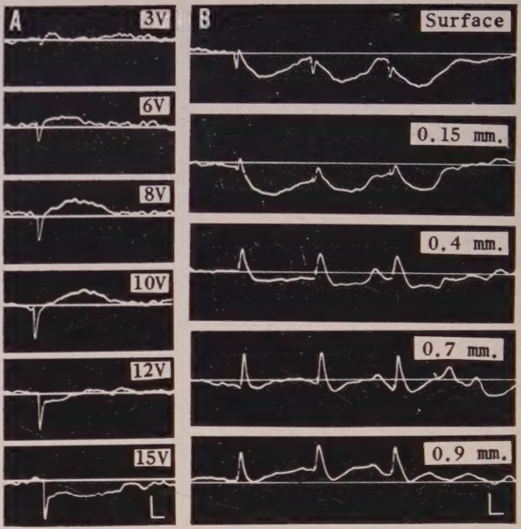

FIG. 1. *A*, cortical responses to stimuli of increasing strength. *B*, serially evoked responses to direct cortical stimulation (6/sec.) as cortical recording electrode is lowered through cortex. Note that primary potential is clearly reversed at 0.15 mm, whereas slow negativity does not show complete reversal until depth of 0.9 mm is reached. Straight line = baseline. Positive up. Vertical cal. line = 500 μV; horizontal, 30 msec.

For Fig. 1 *B*, a saline-filled pipette, having an outside diameter of 100 μ and led from a calomel half-cell, was inserted through the surface cortical layer. The tip was lowered in 0.1 mm fractions (or less) while recording the responses to serial stimulation at 6/sec. The polarity of the potential which we have called *slow negativity* was found to reverse more deeply (0.7 to 0.9 mm) than that of the preceding dendritic one (0.15-0.4 mm).

Pharmacological selectivities also aid in differentiating the 2 components and are well exemplified by the effect of chloral hydrate upon them(6). Fig. 2 *C* and *D* illustrate the potential complex composed of dendritic potential and slow negativity as it is elicited with serial stimulation at 15 V., before and after intravenous administration of a total of 500 mg of chloral hydrate. The summation of slow negativity which occurs upon 20/sec.

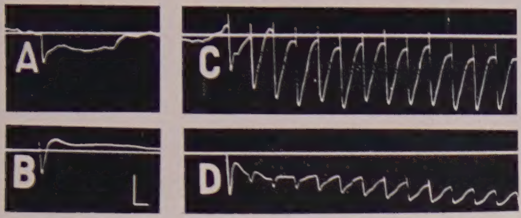

FIG. 2. *A*, cortical response to single stimulus (15 V). *B*, same after intrav. inj. of 150 mg chloral hydrate. *C*, serially evoked responses to direct cortical stimulation (20/sec.). *D*, same as *C*, after intrav. inj. of 500 mg chloral hydrate. Cal., as in Fig. 1.

stimulation becomes exaggerated after the injection (compare Fig. 2 *D* with *C*). By contrast, at the same stimulus frequency the chloral hydrate effects a reduction in the amplitudes of the successively evoked dendritic potentials past the first response of a series.

Fig. 2 *A* and *B* illustrates another effect of chloral hydrate. This one appears following intravenous administration of lesser amounts of the drug (Nembutal behaves similarly). With 150-200 mg injections the slow negativity of Fig. 2 *A* is replaced by an augmented positive after-effect. However, that the slow negativity is not lost is indicated by the downward dip in the positively directed deflection which follows the dendritic potential in Fig. 2 *B*. Although with an injection of this amount the negativity persists, it is obscured through algebraic summation with the augmented after-positivity described above. The additional injection of chloral hydrate to 500 mg again reduces the positive after-effect. It is important that the after-positivities illustrated in Fig. 2 *B* and 1 *A* do not summate upon serial stimulation. For this reason as well as for its identity in threshold with the dendritic potential we still view the slow positivity as an after-effect and not as a separate excitatory process.

Discussion. Evidence drawn from pharmacological selectivity as illustrated in Fig. 2 *D*, threshold differences (Fig. 1 *A*), and differences in depth of origin in the cortex (Fig. 1 *B*), support the conclusion that the dendritic potential and the slow negativity which follows in its wake are different processes. Responses indicative of 3 post-synaptic excitation processes of cortex have been identified by Tasaki, Polley and Orrego(7): axonal responses, duration 1 msec., cell body responses 1.5-3. msec., and dendritic response, 15 msec. We suggest that the 150-300 msec. slow negative wave which we describe represents still another post-synaptic phenomenon. The fact that the stimulus used is a very local one and recording is accomplished within 0.5 mm of the stimulus site argues against an origin by fusion of many temporally dispersed short-lasting processes of variable latencies. The fact that the slow negativity exhibits graded response qualities suggests a dendritic source (3). No other possibilities are apparent at this time.

A unique feature of the slow negativity we describe is its summation during serial stimulation and the continuance of the disturbance past the end of a stimulus period (1-3 sec.) The last indicates singularly slow recovery. Both of these characteristics are evident when the slow negativity is excited from midline thalamus(8). This suggests that the activity of which it is the sign plays a definite role in cortical functioning.

Summary. On the basis of threshold differences, depth of origin in cerebral cortex and pharmacological selectivity, we conclude that 15 msec. negative potential assignable to activity in apical dendrites and ensuing 150-300 msec. slow negative wave are separate neural processes. Our evidence suggests that the slow negativity seen in the wake of dendritic potential is a sign of an independent post-synaptic process having unusually long time course.

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